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THE DISTRIBUTION OF CALCIUM GIVEN TO HUMAN SUBJECTS BY SUSTAINED INTRAVENOUS INFUSION

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ABSTRACT

Solutions of calcium gluconate which contained known amounts of Ca^{45} were given to human subjects by intravenous infusion (usually 110 mg. of calcium per hour for four hours). Because the specific activity of the infused calcium was known, its subsequent distribution and excretion could be followed. It was found that the infused calcium left the plasma rapidly. The approximate amount of calcium into which the administered calcium appeared to mix was calculated. This varied between 1.5 and 3.1 Gm. in patients with osteoporosis, was 4.1 Gm. in a patient with a parathyroid adenoma and 7.3 Gm. in a subject with Paget's disease. Although the amount of calcium excreted in the urine on the day of the infusion was greatly increased in all patients (equal to 34–98 per cent of the amount of calcium administered) most of the excreted calcium was from sources other than the infusion. From 1.6 to 13.0 per cent of the infused calcium appeared in urine during the first twenty-four hours.

THE responses of normal human beings to infusions of calcium gluconate include hypercalcemia, hyperphosphatemia, hypercalciuria and reduction in the rate of excretion of phosphate. This last response, which is the only one to have been studied extensively, is presumed to be a reflection of a reduced parathyroid hormone effect in subjects whose parathyroid glands react normally to hypercalcemia (1), and has formed the basis of a test which has proved to be of use in the management of subjects with parathyroid diseases (2–4). In contrast, much less is known about the factors which influence the development of hypercalcemia and hypercal-

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ciuria, and attempts to demonstrate characteristic abnormalities of these responses in subjects with skeletal diseases have been less successful (4–6). The present studies were undertaken to see whether these factors could be better defined and whether it was possible to relate variations of the responses to different states of bone metabolism. To these ends, subjects with either normal or accelerated rates of bone formation were given infusions of calcium gluconate which contained radioactive calcium (Ca^{45}). The subsequent distribution of the administered calcium ions was then calculated by comparison of the radioactivity of the infused calcium with that of the calcium of blood and urine. It was shown that the infused calcium mixed rapidly with an amount of calcium which was larger than that in extracellular fluid but less than the total amount of exchangeable calcium of the body, and that this amount was greater in patients with accelerated rates of bone formation than in those whose rates of bone formation were normal.

CLINICAL MATERIAL AND METHODS

One or more tests were carried out in each of 7 patients, all of whom were admitted to the hospital and given a diet which contained 110 mg. of calcium and 600 mg. of phosphorus for at least five days before, and on the day of the test. Five patients had osteoporosis (of these, 1 had disuse atrophy following immobilization of a leg, and 1 had nonactive acromegaly as well as osteoporosis). One patient, subsequently shown to have a parathyroid adenoma, had had hypercalcemia for at least six months; serum alkaline phosphatase activity was 3.6 to 5.9 Bodansky units per 100 ml. and there was slight x-ray evidence of demineralization but not of osteitis fibrosa cystica. The seventh patient had widely distributed and extremely active Paget's disease (serum alkaline phosphatase activity, 109 King-Armstrong units per 100 ml.).

The tests were carried out in fasting patients, as follows. An infusion of calcium gluconate-glucoheptonate was given by vein, usually at the rate of 110 mg. of calcium (in 100 ml. of isotonic sodium chloride solution) per hour for four hours. Known amounts of Ca^{45} (usually 2 to 5 μc.) were added to the infusions.[1] For some studies, the rate (controlled by a peristaltic infusion pump) and duration of the infusion were varied. Blood samples were taken periodically by venipuncture or from an indwelling catheter. Complete collections of urine were made on the two days before the test and on the day of the infusion. In some studies, collections of urine and stools were made also for the succeeding five to ten days. The concentrations of phosphorus in plasma and urine were determined by the method of Fiske and SubbaRow (7), and creatinine in urine by the method of Peters and Van Slyke (8). Calcium was measured by the method described subsequently, and the Ca^{45} content of plasma and urine was determined by the procedure used previously (9) except that all samples of plasma were ashed (10) to remove organic material before precipitation of calcium oxalate.

Calcium analysis. Calcium was measured by a method similar to that of Munson *et al.* (11) but with several modifications which permitted its application to urine and to solutions prepared from ashed homogenates of stool or diet. Measured aliquots of urine (or

[1] Obtained from the Oak Ridge National Laboratory on allocation from the United States Atomic Energy Commission.

other solutions with a high concentration of phosphate) estimated to contain between 0.02 and 0.10 mg. of calcium were placed in conical 12-ml. centrifuge tubes[2] and diluted with water[3] to a volume of about 5 ml. The pH was adjusted to 4.5 by addition of dilute ammonium hydroxide or acetic acid (methyl red, 0.1 per cent in 50 per cent ethanol, as indicator). Then 2.5 ml. of a 3 per cent solution of ammonium oxalate was added, and the sample was mixed and allowed to stand overnight, after which 2 drops of 2 per cent Triton N.E.[4] were added (to reduce surface tension) and the precipitate was collected by centrifugation. The supernate was discarded by rapid inversion of the tubes, which were kept inverted until after they had drained for five minutes. The precipitate was dissolved in 2 drops of 6*N* HCl and the sides of the tubes washed with water, after which 2.5 ml. of 2*N* NaOH and 0.2 ml. of .025 per cent corinth[5] Ca (12) were added. Titration was carried out (within twenty minutes) in a Coleman Jr. spectrophotometer set at 610 mμ and the end-point determined by plotting successive changes of optical density after additions of known amounts of 0.018 per cent disodium,di-hydrogen-ethylene-diamine-tetra-acetic acid. The concentration of calcium in plasma or serum was measured by the same titration but without deproteinization or previous oxalate precipitation. Foaming of plasma or serum was suppressed by a small drop of caprylic alcohol. Replicate analysis of 42 samples (18 plasma, 20 urine and 4 solutions of ashed stool homogenates) by this and a reference method (10) showed satisfactory agreement. The mean concentration of calcium in plasma from normal subjects was 9.56 ± 0.45 mg. per 100 ml.[6] when determined by the method described, and 9.30 ± 0.60 mg. per 100 ml. by the reference method. The difference between the means (of all samples) was 2.0 per cent of the mean, and the standard error of the mean was 0.73 per cent.

Calculations. The specific activity of calcium in plasma, urine and stools was expressed as a percentage of the dose of Ca^{45} per Gm. of calcium. These data were used to calculate the rate of accretion of calcium in the skeleton and the total amount of exchangeable calcium of the body (E_T). The calculations, which were made as described by Bauer, Carlsson and Lindquist (13, 14), rest on the assumption that the observed fall of specific activity after administration of Ca^{45} can be accounted for by two simultaneous processes: 1) mixing of the Ca^{45} with a quantity of unlabeled calcium of the body (the E_T) and, 2) replacement by unlabeled calcium of calcium lost from the E_T because of excretion in stools and urine and because of accretion. In this study the calculations were carried out after Ca^{45} mixed with about 440 mg. of unlabeled calcium had been given over a period of several hours instead of as a tracer dose given rapidly.

For studies of the distribution and excretion of infused calcium, the proportion of calcium (in samples of calcium from plasma and urine) derived from the atoms of calcium previously infused was calculated as follows:

mg. infused calcium/Gm. calcium = mg. calcium infused (100% dose of $Ca^{40}+Ca^{45}$) $\times$ specific activity (% dose Ca^{45}/Gm. calcium).

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[2] Pyrex catalogue No. 8500.

[3] Glass-distilled or deionized water used throughout the procedure.

[4] Rohm and Haas Co., Philadelphia, Pa.

[5] Corinth Ca is di-sodium-1-hydroxy-4-chloro-2,2-diazobenzene-1,8-di-hydroxy-naphthalene-3,6-disulfonic acid, obtained from Clinton Laboratories, 6010 Wilshire Blvd., Los Angeles, California. In contrast to murexide, this dye is stable in neutral solution for two months and in NaOH during the titration.

[6] Standard deviations.

This calculation could be made because of the presumption that the 2 isotopes of calcium present in the infusions were subject to identical processes of distribution and excretion.

RESULTS

As shown in Table 1, all subjects excreted more calcium in the urine on the day of the infusion than on the previous day. Excretion was increased by an amount equal to a substantial fraction (34 to 98 per cent) of the total quantity of calcium infused. The isotope data show that only 1.6 to 13.0 per cent of the calcium atoms infused were excreted in the urine on the first day, and therefore that most of the excreted calcium was derived from sources within the body. The responses of the 2 patients (one with osteoporosis, the other a parathyroid adenoma) are further illustrated by Figure 1, in which the rates of excretion of calcium and phosphorus are compared. It can be seen that, even during the period of infusion when hypercalcemia was most marked, the rate of excretion of infused calcium was a great deal slower than the rate of infusion, and therefore that the administered calcium must first have mixed with a relatively large amount of endogenous calcium and then been excreted (with the endogenous calcium) in the urine.

Figure 2 shows that the specific activity of calcium in plasma increased throughout the period of infusion. In most studies, at least during the first three hours of infusion, the specific activity increased by a relatively constant amount each hour.

The number of milligrams of infused calcium per gram of calcium in plasma was calculated (total mg. of calcium infused × per cent of dose of Ca^{45}/Gm. calcium) and, from this, the concentration of infused calcium in plasma (Table 2). In contrast to the total concentration of calcium in plasma (endogenous + infused) which rose for two or three hours and there-

TABLE 1. EXCRETION OF CALCIUM IN URINE ON THE DAY BEFORE, AND ON THE DAY OF AN INFUSION OF CALCIUM GLUCONATE AND Ca^{45}

Test No.*	Calcium infused (mg.)	Total excretion of calcium		Increase in excretion of calcium on day of infusion (mg.)	Excretion of infused calcium on day of infusion	
		day before infusion (mg.)	day of infusion (mg.)		(% of dose)	(mg.)
2	85	91	160	69	7.1	6
3	452	111	554	443	11.2	51
5	376	184	349	165	13.0	49
7	440	164	399	235	8.2	36
8	440	254	658	404	9.5	42
9	440	211	361	150	1.6	7

* Tests 5 and 8 are illustrated by Figure 1.

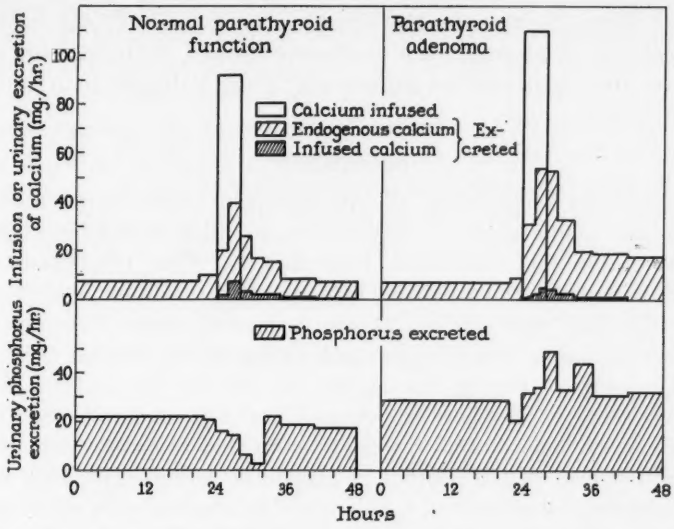

FIG. 1. Comparison in an osteoporotic and a hyperparathyroid patient of the rates of urinary excretion of calcium and phosphate during the day before and the day on which an intravenous infusion of calcium labeled by Ca^{45} was given. The total amount of calcium given, and the amounts of administered calcium and of endogenous calcium excreted in the urine, are each indicated by the area under the appropriate curve.

after remained relatively constant until several hours after the infusion had been stopped, the concentration of infused calcium and the specific activity rose throughout the period of infusion and immediately thereafter

TABLE 2. CONCENTRATION OF CALCIUM IN PLASMA AND CALCULATED QUANTITY WITH WHICH INFUSED CALCIUM APPEARED TO MIX (E_R) AFTER THREE HOURS OF INFUSION

Test No.	Sex & age	Diagnosis	Rate of infusion of calcium (mg./hr.)	Concentration of calcium in plasma (mg./100 ml.)			E_R at 3 hrs. (Gm.)	Calcium of E.C.F.* (Gm.)	E_T† (Gm.)	A† (Gm./day)
				Total Ca		Infused Ca at 3 hrs.				
				0 hr.	3 hrs.					
1	M 55	Osteoporosis	110	8.7	9.5	1.38	2.7	.86	5.1	.29
2			17	9.3	9.3	.32	1.8			
3	F 51	Osteoporosis (disuse)	130	9.8	11.9	1.64	2.1	1.17	2.9	.34
4			110	9.9	10.7	1.29	2.4			
5	F 41	Osteoporosis	94	10.1	11.0	1.75	1.5	.89	4.4	.41
6	F 73	Osteoporosis	40	9.4	9.3	.67	1.9	.85		
7	F 58	Osteoporosis and acromegaly	110	9.6	10.0	1.05	3.1	1.02	6.8	.54
8	M 62	Parathyroid adenoma	110	11.0	11.2	.73	4.1	1.06	8.0	.87
9	M 61	Paget's disease	120	9.3	9.5	.53	7.3	.92	47.0	5.1

* Calcium of E.C.F. calculated from [Kg. body wt. ×Gm. calcium/L. in plasma] ×[.05 +.66 ×.15].

† E_T and A (total exchangeable calcium and accretion rate) are calculated by the method of Bauer, Carlsson and Lindquist (13, 14) modified as described in the text.

decreased. These changes, both of specific activity and of total concentration of calcium in plasma, were much less marked in the patient with a parathyroid adenoma and the patient with Paget's disease than in the patients with osteoporosis.

DISCUSSION

Studies of the distribution of intravenously administered Ca^{45} have added greatly to our understanding of bone metabolism and form the basis for interpretation and evaluation of the data described. It has been shown that Ca^{45} mixes with the unlabeled calcium of extracellular fluid (E.C.F.), intracellular fluid and calcium at the surface of bone. Together, these quantities of calcium comprise the total exchangeable calcium (E_T) of the body. The rates of mixing throughout the E_T are not uniform. For instance, exchange of ions between the calcium of plasma and other E.C.F. occurs at an extremely rapid rate, similar to that of sodium and chloride (52 to 88 per cent of the content of plasma per minute (15, 16)), but exchange between the calcium of E.C.F. and a substantial part of the calcium ions of intracellular fluid is relatively slow (17, 18). The calcium of E.C.F. mixes also with a small part of the calcium of the skeleton. This mixing, which has been shown to proceed at several different rates (19), occurs because of exchange of ions in solution in E.C.F. for ions at the surface of bone crystals which are exposed to E.C.F. Therefore, the amount of exchangeable calcium of bone should depend to some extent on the total surface area of bone crystals in contact with E.C.F. Thus, it is not surprising that in fully calcified bone (where crystals are so densely packed that ions cannot migrate freely between them), the amount of exchangeable calcium is small, whereas in newly formed, incompletely calcified bone, exchange reactions are much more active (20). These considerations are supported by the observed correlation in human beings between the rate of bone formation and amount of E_T (21) and by the demonstration, by radioautography, that exchange reactions are most intense in areas of recently formed, incompletely calcified bone (22). Calcium ions leave the E_T by excretion (urine, stools and sweat; about 0.3 Gm. per day in normal subjects) and by accretion (approximately the same as new bone formation and shown to equal about 0.5 Gm. per day in normal adult subjects (14, 21)).

The rate of accretion and E_T was calculated for all but 1 of the subjects with osteoporosis (Table 2). These values were comparable to those reported for other patients with osteoporosis, and also for subjects of similar age, but without evidence of osteoporosis (14, 21). In contrast, the hyperparathyroid patient and the subject with Paget's disease were each shown to have accelerated accretion and increased E_T typical of the values reported in studies of similar patients (9, 21).

At least during the first three hours of infusion, the specific activity of calcium in the plasma of the patients with osteoporosis increased by a relatively constant amount per hour (Fig. 2). This suggests that, for the most part, the infused calcium mixed with a quantity of calcium of the body and that, once mixed, it was not replaced significantly by unlabeled calcium during the period of infusion. The quantity or "pool" of calcium into which it appeared to mix can be estimated from the following relationship: pool at time t = (amount of calcium given from time 0 to time t)/(specific activity of calcium in pool at time t). This quantity of calcium, which is

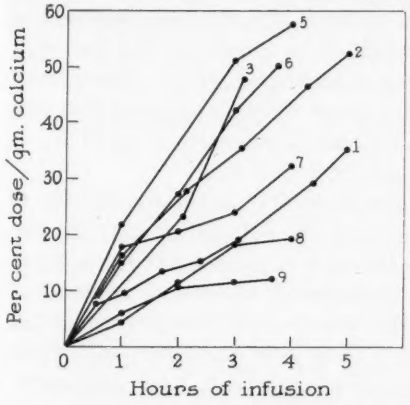

FIG. 2. Specific activity of calcium in plasma of all subjects studied. The test numbers correspond to those in Tables 1 and 2 and Figure 3.

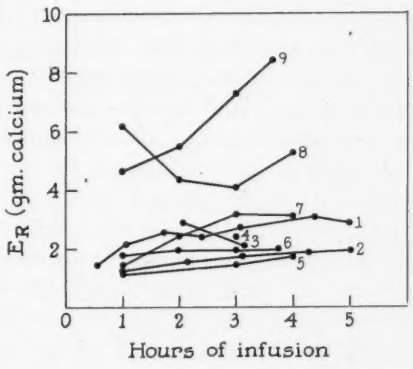

FIG. 3. Quantity of calcium in the body with which calcium given by infusion appeared to mix (E_R). The test numbers correspond to those in Tables 1 and 2 and Figure 2.

necessarily part of the E_T, will be referred to as the rapidly exchangeable calcium (E_R). The E_R's calculated from the data in Figure 2 are shown in Figure 3, and the E_R's at the end of the third hour of infusion are indicated in Table 2. It can be seen from Table 2 that in every case the E_R was greater than the total quantity of calcium in the E.C.F., but less than the E_T. This suggests that the E_R consists of the calcium of E.C.F., part of the intracellular calcium, and the most rapidly exchanging calcium of bone.

The E_R could be accurately calculated only if mixing of infused calcium with E_R were rapid relative to the rate of infusion (so that the specific activity of all parts of the E_R were the same), and if there were neither significant addition of unlabeled calcium to, nor loss of calcium from the E_R between time 0 and time t. In view of the rate of mixing of plasma calcium with that of other E.C.F. (approximately 11 to 19 Gm. per hour according

to the figures already quoted), it seems reasonable to suppose that the rate of mixing within the E_R is much more rapid than the rate of infusion (0.11 Gm. per hour). The second assumption cannot be so easily supported and limits the accuracy of this calculation. Calcium can leave the E_R because of excretion and accretion—together equal to about 33 mg. per hour (0.8 Gm. per day) in normal subjects, and probably no more than 100 mg. per hour in most abnormal patients. This loss per hour would be equal to only a few per cent of the E_R. However, the rate of mixing of the calcium of the E_R with other calcium of the E_T may be more significant. The effect of such mixing would be to decrease the specific activity of the calcium of the E_R (because of exchange of labeled calcium of the E_R for unlabeled calcium of the E_T) and thus to cause the calculated E_R to be too great. The fact that there was relatively little change in the calculated E_R for at least three hours in the studies of patients with osteoporosis (Fig. 3) suggests that this mixing is not rapid enough to affect significantly the calculation of E_R in patients with normal E_T's and normal rates of accretion.

In spite of the fact that the E_R can be estimated from these data only in qualitative terms, several facts indicate that this calculation may furnish reproducible information which reflects the state of bone metabolism. First, the calculated E_R remained relatively constant for at least three hours of infusion in the studies of patients with osteoporosis. Second, when the same patient was studied twice, similar values were calculated each time. Third, the calculated E_R's of the osteoporotic patients were reasonably similar ($2.2 \pm .6$ Gm. calcium) and adequately reflected their metabolic state, as indicated by the calculated accretion rates and E_T's. Fourth, although the E_R of the 2 subjects with accelerated rates of accretion could not be estimated precisely, the calculated value, like the E_T, was moderately elevated above control values in the hyperparathyroid subject and greatly increased in a patient with Paget's disease. These findings support the conclusion that the E_R, like the E_T, is increased in conditions associated with accelerated accretion, presumably because both the E_R and the E_T are contributed to significantly by the exchangeable calcium of bone.

The factors which govern the excretion of calcium (infused+endogenous) in urine appear to include the amount of calcium infused, the amount of calcium of the body with which the infused calcium mixes, and the relative effectiveness of various homeostatic mechanisms (including excretion by the kidneys) which tend to reduce the concentration of calcium in the E_R. Thus a given dose of calcium caused a much greater proportionate increase in the amount (and therefore concentration) of calcium in the E.C.F. of the patients with small E_R's than in those with large E_R's. The resulting hypercalcemia brings into play those mechanisms which tend to return the concentration of calcium to normal. If mechanisms other than excretion,

such as a further distribution by mixing with other exchangeable calcium (also theoretically possible, is formation of new bone crystals or reduction in the rate of bone resorption) are effective, the hypercalcemia will be of short duration and a relatively small amount of extra calcium will be excreted in the urine. These effects are seen in tests of patients with osteomalacia in whom large amounts of uncalcified bone matrix are present (6) and in the study reported here of a subject with Paget's disease whose E_T was very large and in whom the rate of mixing between calcium of E_R and E_T appeared to be rapid. When such mechanisms are not effective (as in subjects with small E_R's and E_T's), a relatively larger amount of calcium will be excreted in the urine.

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THE EFFECT OF PITUITARY STALK SECTION ON THE ADRENAL FUNCTION OF WOMEN WITH CANCER OF THE BREAST*

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ABSTRACT

Pituitary stalk section was performed in 26 women with rapidly advancing metastatic carcinoma of the breast. A tantalum plate was inserted above the sella to retard revascularization from the hypothalamus. Summarized results of detailed studies of adrenal function both before and after the operation are presented. Many of the patients excreted low or low-normal quantities of 17-ketosteroids and 17-hydroxycorticosteroids prior to surgery. Following the operation, steroid excretion gradually decreased to the low levels observed in hypopituitarism and characteristic symptoms of adrenal insufficiency developed. These symptoms were alleviated by the administration of ACTH or prednisolone. When substitution therapy was withheld, there was no tendency for urinary steroids to rise and symptoms of adrenal insufficiency recurred. The capacity of the pituitary-adrenal axis to respond to "stress" was assessed by measuring the rise in the concentration of plasma 17-hydroxycorticosteroids during an insulin-glucose tolerance test and after the administration of bacterial pyrogen. This response was compared with the response to ACTH. The postoperative response to ACTH was frequently diminished or absent, suggesting that varying degrees of adrenal atrophy had occurred. The response to hypoglycemia or pyrogen, however, was usually as great as that obtained by ACTH. The persistence of the stress response in humans with pituitary-stalk section is discussed in the light of present knowledge regarding the hypothalamic control of ACTH secretion.

IT IS now well established that a modification of the hormonal environment may induce clinical improvement in certain patients with metastatic mammary carcinoma. Remissions in a variable percentage of cases have been reported following castration, adrenalectomy, androgen or estro-

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gen therapy, or hypophysectomy (1, 2). Although hypophysectomy has been reported to be effective in inducing clinical remission in over 50 per cent of patients (3, 4), this procedure has a number of disadvantages; total hypophysectomy is a difficult neurosurgical procedure and removal of the pituitary body is frequently incomplete (5). Concern over the potential postoperative morbidity and neurologic sequelae has discouraged some physicians from recommending this procedure.

There is a growing body of experimental data which indicates that the secretions of the anterior pituitary body as well as those of the neurohypophysis are dependent upon anatomic connections with the hypothalamus by way of the pituitary stalk. The hypothalamic-pituitary portal system of veins is thought by some to transport neurohumoral substances which regulate the secretory activity of the anterior pituitary (6). For these reasons, investigations were undertaken in 1955 to determine whether surgical isolation of the pituitary from its hypothalamic connections would prove an effective substitute for hypophysectomy in the management of patients with mammary carcinoma (7). In this procedure, following division of the stalk, a tantalum plate was inserted above the sella extending from the tuberculum sella to the dorsum sella and laterally to each carotid artery. It was hoped that this occlusive barrier would effectively retard regrowth of the portal circulation between the median eminence and anterior lobe of the pituitary.

It was immediately apparent that these patients afforded an unusual opportunity to determine in the human the degree of pituitary activity which is dependent upon direct anatomic connections with the hypothalamus. Therefore detailed studies of adrenal function, thyroid function, gonadotropic hormone excretion, and electrolyte and water metabolism were carried out both before and after surgery. Although the chronically ill patients studied were not ideal experimental subjects, our findings were adequate for certain comparisons with the results of similar investigations in experimental animals and with the reported effects of hypophysectomy on endocrine functions in man. The present paper summarizes the effect of pituitary-stalk section on adrenal function. Subsequent papers will report in greater detail the clinical histories, postoperative course, findings at autopsy, and the effects of the operation on thyroid function, gonadotropin excretion, and electrolyte and water metabolism.

MATERIALS AND METHODS

Twenty-six females between the ages of 32 and 69 with advanced metastatic cancer of the breast were selected for the operation. All of them had rapidly advancing metastatic disease and most of them had been treated previously with one or another form of therapy such as mastectomy, androgens, estrogens, surgical castration, or irradiation of the ovaries. In 24 of the patients the stalk was thought to be completely severed and

the tantalum plate was shown by x-ray examination to be properly located above the sella; in 1 (No. 23) the stalk was incompletely severed and postoperative films showed the plate to be displaced. Section of the pituitary stalk appeared to influence favorably the course of metastatic breast carcinoma in some of these patients (7); in a few, the improvement was dramatic.

Serial 24-hour urine collections were obtained on most patients during the initial and subsequent admissions to the hospital. Urinary 17-ketosteroids were determined by a modification of the methods of Drekter *et al.* (8) and Wilson (9), after sulfuric-acid hydrolysis in a boiling water bath for thirty minutes. Urinary 17-hydroxycorticosteroids were measured by a modification of the Glenn and Nelson method (10). Interpretation of the urinary data is complicated by the fact that it was impossible to carry out prolonged observations without the modifying influence of adrenal substitution therapy. The excretion of large volumes of urine due to diabetes insipidus, or of small volumes of urine during periods of water retention, further complicated the interpretation of urinary steroid excretion data in the postoperative period. A typical patient was given 200 mg. of cortisone parenterally on the day of operation. This dosage was rapidly tapered over the first few postoperative days and thereafter prednisolone was given in a dosage of 5 to 10 mg. daily. Prednisolone was chosen for substitution therapy since, in the dosage given, it in itself contributes very little to the measurable urinary steroids. Some patients were given adrenocorticotropin (ACTH) postoperatively, and a few of the later ones received no substitution therapy until signs of adrenal insufficiency developed. Postoperative studies were commenced as soon as the patient was over the immediate effects of the surgery and these tests were repeated at intervals thereafter.

The response of the free and conjugated plasma 17-hydroxycorticosteroid levels to a six-hour infusion of 25 international units of ACTH was determined in both the preoperative and postoperative periods. In some patients the ability of the adrenal to respond to ACTH was compared with its response to a standard "stress test." The stresses used were insulin hypoglycemia and bacterial pyrogen. The free 17-hydroxycorticosteroid levels in plasma were measured by a modification of the method described by Nelson and Samuels (11). The conjugated corticoids were determined by a modification of the Bongiovanni and Eberlein method (12). The results have been expressed as total plasma 17-hydroxycorticosteroids.

Insulin-glucose tolerance test. At 9:00 a.m., blood was drawn for the determination of fasting blood sugar and plasma free and conjugated 17-hydroxycorticosteroids. Insulin in a dosage of 0.1 unit per Kg. was administered intravenously and thirty minutes later glucose was given either by mouth in a dosage of 1.75 Gm. per Kg. or intravenously in a dosage of 0.5 Gm. per Kg. (13). Blood sugar and plasma corticoid levels were determined at intervals up to three hours following the injection of insulin. Blood sugar was determined by the Nelson method (14).

Bacterial pyrogen test. After drawing blood for determination of 17-hydroxycorticosteroids, Piromen[1] in a dosage of 0.5 μg. per Kg. was infused intravenously over a five-minute period (15). A temperature curve was plotted and blood corticoid concentrations were determined at various times up to three hours after the infusion. An attempt was made to perform the ACTH and "stress" tests as soon as possible in the postoperative period. In all but a few instances, treatment with cortisone or prednisolone was discontinued during these tests. In a few cases the adrenal was "primed" for a few days with

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[1] A polysaccharide derived from pseudomonas species (Travenol Laboratories, Inc., Morton Grove, Illinois).

ACTH before the test was performed. In a later group of patients these tests were made before substitution therapy was instituted.

RESULTS

Urinary excretion of steroids

In every patient the excretion of 17-ketosteroids and 17-hydroxycorticosteroids was markedly diminished during the period of prednisolone therapy. Exogenous corticoid medication notoriously reduces the secretion of adrenal steroids by inhibition of the normal ACTH mechanism, but it was our impression that the disappearance of 17-ketosteroids and 17-hydroxycorticosteroids from the urine in these patients was more complete than that usually observed in normal patients given this amount of exogenous steroid. Unfortunately, strictly comparable data in similarly treated patients with intact pituitary stalks are not available.

When substitution therapy was withdrawn the steroid excretion remained at a low level and there was no tendency for the urinary steroids to rise during the withdrawal period. In Figure 1, the preoperative 17-keto-

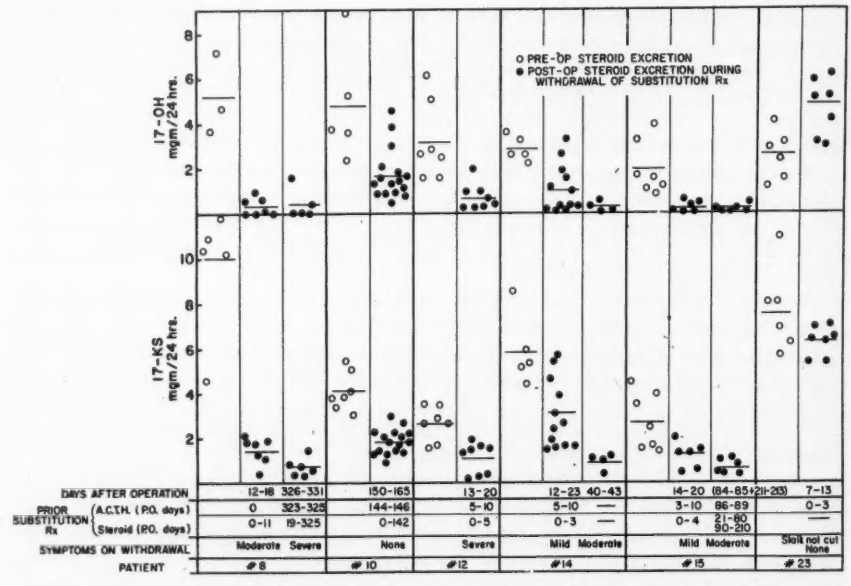

FIG. 1. Excretion of 17-ketosteroids and 17-hydroxycorticosteroids in 6 representative patients before operation and at various times postoperatively when substitution was withheld. The substitution therapy prior to each period of withdrawal is indicated at the bottom of the chart. The horizontal bar in each column indicates the arithmetic mean of each group of determinations.

steroid and 17-hydroxycorticosteroid excretion in 6 representative patients is compared with that during one or more periods postoperatively when substitution therapy was withdrawn. Patient No. 10 was unique in that she tolerated substitution withdrawal well and on a few days she excreted normal quantities of 17-hydroxycorticosteroids. Paper chromatograms of the urine on these days indicated the presence of tetrahydro E and smaller amounts of tetrahydro F. Patient No. 14 was treated with 12.5 I.U. of ACTH intramuscularly every eight hours between the fifth and tenth postoperative days. She excreted large quantities of steroids during this period. During the following thirteen days her steroid excretion fell slowly to basal levels. During the subsequent weeks, signs of adrenal insufficiency slowly developed and by the fortieth day her urine contained barely detectable quantities of 17-ketosteroids and 17-hydroxycorticosteroids. Patient No. 23, who underwent essentially a sham operation, was treated with ACTH for the first three postoperative days. Between the seventh and thirteenth postoperative days there was no significant diminution in the excretion of 17-ketosteroids as compared with the preoperative levels, and 17-hydroxycorticosteroid excretion had increased significantly.

Many of the patients in this series excreted low or low-normal quantities of steroids prior to operation (a finding which has previously been noted in other patients with advanced cancer (16)). It is conceivable that the pituitary-adrenal mechanism in these instances can be more easily suppressed by small amounts of steroids or ACTH than is normally the case, and that clinical symptoms of adrenal insufficiency will ensue in such patients when suppressive therapy is withdrawn. Although progressively less substitution therapy was used in succeeding cases, it appeared that the effect of pituitary-stalk section on adrenal function should be further assessed in patients who were supported throughout the immediate postoperative period without the use of exogenous steroids and ACTH. The excretion of steroids in the preoperative and immediate postoperative periods in this group is plotted in Figure 2. It is apparent that there was no abrupt drop in the excretion of 17-ketosteroids and 17-hydroxycorticosteroids, and in several patients there was a temporary increase followed by a progressive fall during the subsequent days to lower levels than before operation. As the excretion of these substances diminished toward basal values, clinical signs of adrenal insufficiency developed which prevented further prolongation of the period without substitution therapy. A similar pattern of response has been reported previously in 1 case following pituitary-stalk section (17), and in some cases following incomplete hypophysectomy (18). In totally hypophysectomized patients the fall in urinary steroids is more dramatic (17).

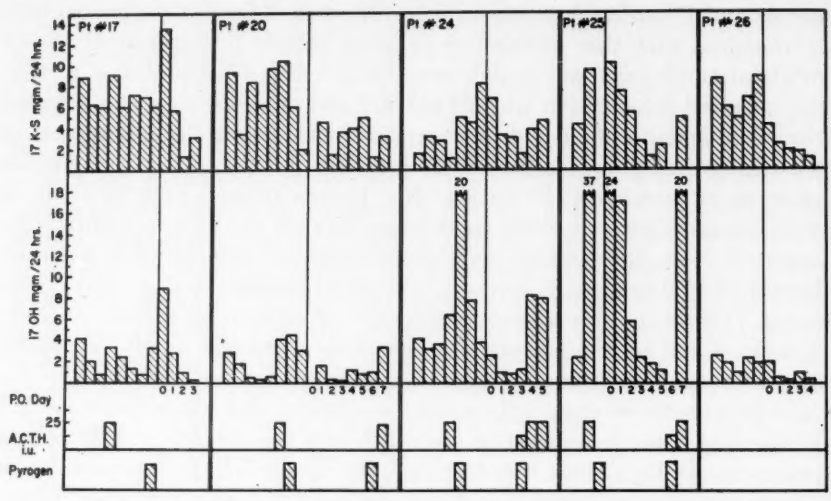

FIG. 2. Excretion of 17-ketosteroids and 17-hydroxycorticosteroids in 5 patients who received no substitution therapy in the immediate postoperative period.

Effect of surgery on plasma corticoids

From the findings in the patients with a prior history of steroid therapy and in those who received no substitution therapy during the immediate postoperative period there would appear to be little doubt that endogenous adrenal secretion is diminished to a low level following transection of the pituitary stalk. The length of time required for steroid excretion to reach a lower level following section of the pituitary stalk suggests that either steroids continue to be secreted in response to the stresses of the first few postoperative days, or that there is delayed excretion of the large amounts secreted during the anesthesia and the surgical procedure preceding actual division of the stalk. To investigate this point further, serial plasma 17-hydroxycorticosteroid values were obtained on 4 patients on the day of operation (Fig. 3). One patient (No. 24) clearly had a higher blood corticoid level twelve hours following section of the stalk than at the actual moment of division. This patient had had higher levels of 17-hydroxycorticosteroids in her blood and urine prior to operation than many other members of this series and she had exhibited a brisk response to ACTH and pyrogen (Figs. 2 and 6). Despite her apparent ability to respond further to stress following transection of the stalk, severe adrenal insufficiency developed on the third postoperative day. Three other patients exhibited a fall in their blood corticoid levels following division of the stalk. The reason for this variation among patients is not clear, but the behavior of the 3 patients whose plasma

corticoid levels fell following division of the stalk deviates markedly from the usual pattern of progressively rising plasma 17-hydroxycorticosteroid levels following major surgery (19).

Insulin-glucose tolerance test

Additional information regarding the ability of "stalk sectioned" patients to respond to physiologic stress was obtained by measuring the plasma corticoid response to insulin hypoglycemia and bacterial pyrogen. Use of the insulin-glucose tolerance test permitted independent study of the homeostatic mechanisms involved in the blood sugar responses to hypoglycemia and comparison of this response with a direct measurement of the

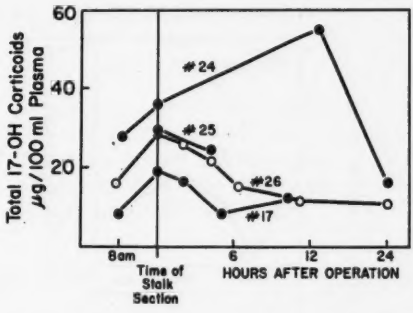

FIG. 3. Plasma 17-hydroxycorticosteroid levels on the day of operation.

adrenal response to hypoglycemia. Following the administration of insulin to normal subjects the blood sugar concentration rebounds within ninety minutes to a level in excess of that in the fasting state (13). We have observed in a group of healthy adults that a rise in the plasma 17-hydroxycorticosteroid level occurs in those in whom the characteristic signs and symptoms of hypoglycemia develop during this test. Occasionally there may be no measurable adrenal response in normal subjects in whom appreciable clinical manifestations of hypoglycemia fail to develop, even though the blood sugar curve rebounds normally.

Typical responses of the blood sugar and 17-hydroxycorticosteroid levels are plotted in Figure 4. Patient No. 14 was tested twice during the postoperative period. On the first occasion, shortly after a period of priming with ACTH, the blood sugar response to hypoglycemia was normal and there was a significant rise in the concentration of plasma 17-hydroxycorticosteroids. After an additional period without further therapy the blood sugar level failed to rebound and there was no plasma corticoid response. At this time the daily excretion of steroids was very low (Fig. 1) and the adrenal was likewise unresponsive to ACTH.

Six of the 12 patients tested postoperatively with the insulin-glucose

tolerance test exhibited on at least one occasion the unresponsive blood sugar curve characteristic of adrenal insufficiency or hypopituitarism. Simultaneous plasma corticoid levels were determined in 5 of these 6 patients and in none of them was a rise in plasma corticoid values observed, although pronounced symptoms of hypoglycemia were present. These abnormal results were probably due to adrenal atrophy, since these patients were likewise unresponsive to ACTH at this time.

A definite rise in the level of plasma corticoids was observed in 5 of the 6 corticoid tests which were carried out on those patients who exhibited a normal blood sugar response to hypoglycemia. The patient who demonstrated no adrenal response likewise was unresponsive to ACTH at this

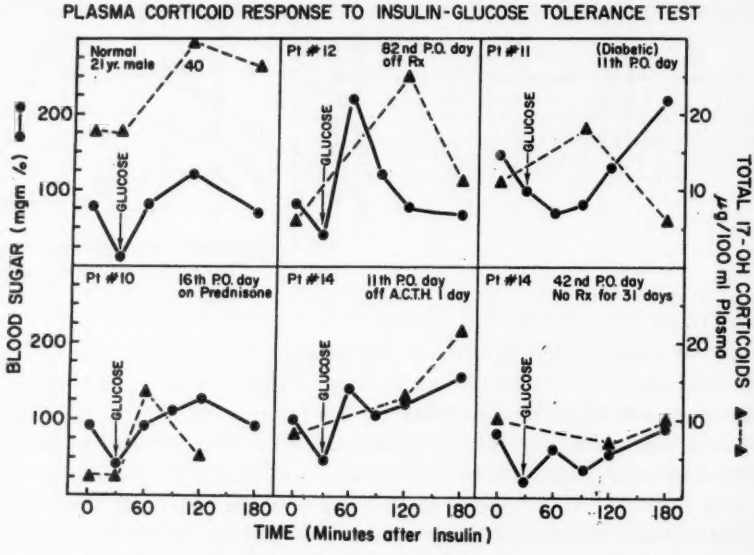
PLASMA CORTICOID RESPONSE TO INSULIN-GLUCOSE TOLERANCE TEST
Normal 21 yr. male
Pt #12
82 nd P.O. day off Rx
Pt #11
(Diabetic) 11th P.O. day
Pt #10
16th P.O. day on Prednisone
Pt #14
11th P.O. day off A.C.T.H. 1 day
Pt #14
42 nd P.O. day No Rx for 31 days
GLUCOSE
BLOOD SUGAR (mgm %)
TOTAL 17-OH CORTICOIDS γg/100 ml Plasma
TIME (Minutes after Insulin)

FIG. 4. Typical responses of the blood sugar and plasma 17-hydroxycorticosteroid levels to the standard insulin-glucose tolerance test.

time. In this patient the standard dosage of insulin produced only a moderate lowering of the blood sugar level, but since the blood sugar value at ninety minutes exceeded the fasting value, she was classified as "normal" by the criteria used in interpreting the insulin-glucose tolerance test. A diabetic patient responded on two occasions with an elevation of plasma corticoid values despite an abnormal response of her blood sugar level.

These results clearly indicate that an intact stalk is not essential for adrenal stimulation in response to the stress of hypoglycemia. In most instances the blood sugar response to hypoglycemia correlated well with the

adrenal response. The insulin-glucose tolerance test by itself, however, provides an imperfect measure of the integrity of the pituitary-adrenal axis, since the blood sugar response may be normal in the absence of demonstrable adrenal stimulation and an abnormal blood sugar curve may occur despite a definite adrenal response.

Pyrogen test

Because of the unpredictability of the degree of hypoglycemia resulting from a standard intravenous dosage of insulin and because of the relatively small response of the adrenal to this stimulus, later patients were tested with bacterial pyrogen. We have observed in normal people and in dogs

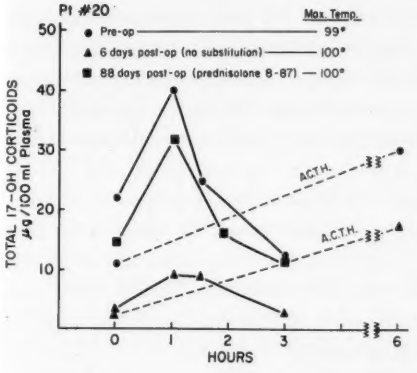

FIG. 5. Patient No. 20. Response of plasma corticoid levels to Piromen (solid lines) and ACTH (broken lines) preoperatively and after stalk section.

with a cannula in the adrenal vein that Piromen in a dosage of 0.5 μg. per Kg. elicits a rise in the level of plasma 17-hydroxycorticosteroids as great as that obtainable with maximal ACTH stimulation.

In 3 patients (Nos. 20, 24 and 25) the response to pyrogen was tested in the early postoperative period prior to the institution of therapy, and this response was compared with the response in the preoperative period. Patient No. 20 (Fig. 5) responded briskly to both pyrogen and ACTH in the preoperative tests, but by the sixth postoperative day the adrenal response to both pyrogen and ACTH was greatly reduced. It would appear, however, that the response to pyrogen was nearly as great as the response to ACTH. On the eighty-eighth postoperative day the adrenal response to both ACTH and pyrogen was similar to the responses in the preoperative period even though substitution therapy had been continuously administered. It is possible that during this interval revascularization of the pituitary or regeneration of the portal system around the tantalum plate had occurred.

Preoperatively, Patient No. 24 (Fig. 6) responded maximally to pyrogen

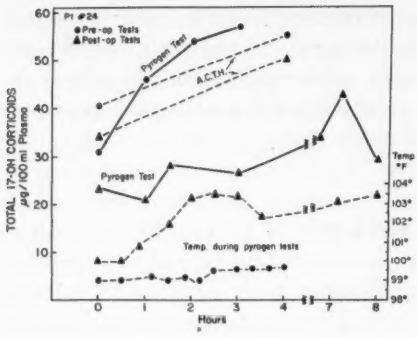

FIG. 6. Patient No. 24. Comparison of the plasma corticoid responses to pyrogen (Piromen) and to ACTH in the preoperative period and three days after section of the pituitary stalk.

as compared with her response to ACTH, but on the third postoperative day the adrenal response to pyrogen was delayed and of smaller magnitude than her response to ACTH later that evening. Although her postoperative response to pyrogen was submaximal, a significant rise in the concentration of plasma corticoids occurred even though clinical signs of adrenal insufficiency were rapidly developing.

Patient No. 25 (Fig. 7) likewise had a brisk adrenal response to pyrogen in the preoperative period. When she was tested again on the fourth postoperative day, an alarming degree of hyperpyrexia developed and cortisone was given one hour after the pyrogen was administered. At this time, however, her adrenal response was as great as it had been one hour after administration of the pyrogen during the preoperative test.

Two other patients exhibited a definite response to pyrogen in the postoperative period, but quantitatively the response was smaller despite a greater degree of fever during the second test. In each patient the postoperative response to ACTH was similarly diminished. Of the 8 patients

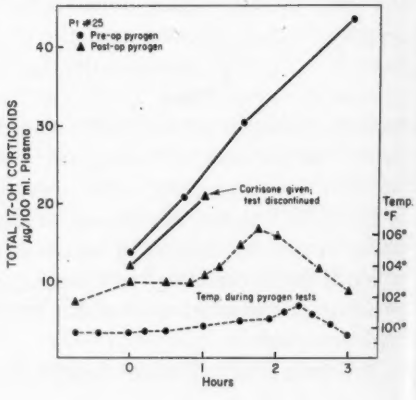

FIG. 7. Patient No. 25. Plasma corticoid response to pyrogen (Piromen) in the preoperative period is compared with that on the fourth postoperative day. Only the one-hour response is plotted postoperatively, since cortisone was administered for treatment of the hyperpyrexia which developed.

tested postoperatively with bacterial pyrogen, 7 responded with an unequivocal rise of the level of plasma 17-hydroxycorticosteroids. In most instances the rise was comparable to that produced by ACTH. The eighth patient, who exhibited no rise, was likewise unresponsive to ACTH.

Clinical symptoms

During the period of steroid withdrawal, unmistakable clinical symptoms of adrenal insufficiency, consisting of fever, hypotension, lethargy and anorexia, developed to a variable extent in a majority of the patients (Table 1). There was marked variation in the promptness with which these symptoms appeared and in their severity, but each patient experienced a dramatic improvement in the feeling of well-being when therapy with adrenal steroids or ACTH was reinstituted. The ability to tolerate withdrawal appeared to depend on the acuteness of the underlying disease. For example, in Patient No. 3 (who in the hospital experienced only mild symptoms on withdrawal of therapy), the characteristic picture of adrenal insufficiency developed at home during the course of an infection when she failed to take her prednisolone. She died during what was probably an adrenal crisis.

DISCUSSION

It is our assumption that section of the pituitary stalk with the insertion of a tantalum barrier above the sella effectively interrupts direct vascular as well as neural connections between the hypothalamus and the pituitary, at least for a time. Although the nature of the operation and the extent of the tantalum barrier makes this a reasonable assumption, it is doubtful whether final anatomic proof regarding the disruption of all portal connections can be established by using permissible autopsy techniques. Autopsy on one of our patients (No. 21) twelve months following surgery disclosed grossly visible vascular connections posterior to the tantalum plate. This was not unexpected, however, since her endocrine function had returned to normal in the months preceding her death. Smaller vascular connections are easily missed and even under the relatively ideal circumstances of animal experimentation, it is difficult to prove that no portal communications remain or have regenerated following section of the stalk (20).

In the dog, secondary adrenal atrophy does not occur until over 75 per cent of the anterior lobe of the pituitary is removed, if the stalk is left intact (21). Postmortem studies of our patients, to be reported in greater detail later, have so far revealed a substantial amount of surviving viable pituitary tissue and no evidence of infarction of the adenohypophysis. Our data do not permit us to say whether those pituitary functions which were

TABLE 1. SYMPTOMS AFTER WITHDRAWAL OF SUBSTITUTION THERAPY

Patient No.	Postop. day on which Rx discont'd	Duration of withdrawal (days)	Symptoms			Laboratory findings	
			Fever	Weakness, lethargy, anorexia	Hypotension	Glucose-insulin tolerance curve	Blood corticoid response to insulin
				None or Mild			
3*	25	7	+	+	0	Normal	—
	49	7	0	+	+	Abnormal ×2	—
	85	10	0	0	0	—	—
10	147	10	0	0	0	Normal	++
14	11	31	0	++	+	Normal 11th p.o. day Abnormal 42nd p.o. day	++ 0
20	0	6	+	+	0	—	—
21	0	6	0	0	0	—	—
				Moderate			
1	21	9	0	+++	+	Normal	—
12	11	10	0	+++	++	Abnormal	0
	78	3	0	++	+	Normal	++++
15	11	11	—	++	++	Abnormal	0
	81	4	++	+++	++	Normal (previous ACTH)	+
17	0	4	++	+++	++	—	—
	14	4	+	++	++	Normal	0
	86	6	++	+	++	Normal	+
				Severe			
2	4	4	+++	++++	+	—	—
	26	10	++	+++	+++	Normal	—
5	9	7	+	++++	++	Normal (insensitive to insulin pre- & postop.)	—
8	12	7	+	++	+	Normal	—
	106	6	++	++++	++++	Abnormal	—
	326	7	++++	++++	++++	Abnormal	—
24	0	6	+++	++++	++	—	—
25	0	6	++++	+++	++++	—	—
	60	4	++	++++	+++	—	—

* Died at home on the 253rd day, probably in an adrenal crisis.

reduced following this operation were altered by disruption of the portal system of veins, ischemia of the anterior lobe, or by some other mechanism. Those functions which remained intact, however, are of particular interest since in all probability they are not dependent on direct neural or vascular connections between the hypothalamus and the pituitary.

Fortier (22) presented evidence which he interpreted as indicating multiple levels of control over ACTH secretion. He demonstrated that by transplanting the pituitary to the anterior chamber of a rabbit's eye, basal ACTH secretion is diminished and the pituitary loses its ability to respond to psychic stress. He found, nevertheless, that the transplanted pituitary retained its capacity to respond to systemic stress. Recently this author published a critical review of the extensive experimental data pertaining to the hypothalamic control of ACTH secretion and attempted to reconcile the many conflicting observations which have appeared in the literature (23). Our findings, which indicate a persistent adrenal response to strong systemic stressful stimuli in stalk-sectioned humans, are in substantial agreement with the results obtained by a majority of workers who have employed the techniques of stalk section or pituitary transplant in other species. Frequently the postoperative response in our patients was quantitatively less than that observed in the preoperative period, but usually this diminished response could be correlated with a similarly decreased responsiveness of the adrenal to ACTH, indicating that some adrenal atrophy had occurred. We observed no patients who failed to respond to pyrogen or insulin with a rise in the level of plasma 17-hydroxycorticosteroids at a time when a good response to ACTH could be elicited. We made no attempt to measure the adrenal response to psychic stimuli such as apprehension, anger or frustration, since it is difficult to apply these stimuli to humans in a quantitative or reproducible manner.

The mechanism by which the adrenal is able to respond to hypoglycemia or pyrogen in the absence of direct hypothalamic connections is not clear. An intact pituitary is necessary for the adrenal response to pyrogen (24). Fever itself does not appear to be the pituitary stimulant, since the adrenal response often occurs before, or in the absence of a rise in body temperature. Since the rise in corticoid levels following the administration of pyrogen is preceded by a fall in the number of circulating leukocytes, it is possible that circulating leukocyte pyrogen directly stimulates the pituitary to secrete ACTH. Some secondary effect of hypoglycemia acting directly on the pituitary to release ACTH might similarly be postulated to account for the adrenal response to insulin. As an alternative explanation, it is possible that when the portal circulation is disrupted, humoral substances released from the hypothalamus under conditions of acute stress escape into the systemic circulation and reach the pituitary through its arterial supply. Such an interpretation would not deny the importance of the hypothalamus in the pituitary response to stress and would be in agreement with observations in experimental animals indicating that the median eminence must be intact for this response to occur (25).

Although these patients with pituitary-stalk section often retained the

ability to secrete ACTH in response to acute stress, they uniformly had a greatly decreased excretion of adrenocortical steroids and a marked tendency towards the development of clinical manifestations of adrenal insufficiency when substitution therapy was withheld. In these respects our results did not differ greatly from those reported after hypophysectomy (26). We are reluctant to ascribe this functional adrenal atrophy to pituitary ischemia in the absence of infarction, although the possibility cannot be denied. Greer and Erwin (27) demonstrated that the "feed back" mechanism, which controls the daily trophic stimuli to the adrenal, is destroyed by discrete lesions in the hypothalamus. Loss of this mechanism through disruption of the portal system would provide an explanation for the functional and anatomic adrenal atrophy which occurred in these patients. It would be reasonable to assume that if hypothalamic neurohumoral substances regulate the day-to-day basal secretion of ACTH, these substances would be present in low concentration and require direct anatomic connections with the pituitary to exert their effect. Hypothalamic substances concerned with immediate and intense responses, however, would conceivably be secreted in higher concentration and therefore be capable of reaching the pituitary by devious routes through the systemic circulation. Further tests of the "feed back" mechanism in these patients are now being carried out by measuring the response to SU-4885, a substance which inhibits 11β-hydroxylation in the adrenal gland (28).

Acknowledgment

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CARCINOMA OF THE HYPOPHYSIS ASSOCIATED WITH CUSHING'S SYNDROME AND ADDISONIAN PIGMENTATION

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ABSTRACT

A patient suffering from Cushing's syndrome associated with carcinoma of the hypophysis and Addisonian pigmentation is described. It is pointed out that in a patient with Cushing's syndrome, distinct pigmentation may suggest carcinoma of the hypophysis, particularly when combined with an enlarged sella turcica and lack of response to irradiation of the pituitary area.

IN A patient suffering from Cushing's syndrome recently seen in this hospital a carcinoma of the hypophysis was found at autopsy. The patient also showed a characteristic Addisonian pigmentation, and for this reason the case is considered worthy of publication.

CASE REPORT

The patient was a 51-year-old housewife who previously had been in good health. From January 1955 she complained of polydipsia, pigmentation of the skin, particularly of the face, the neck and the abdomen. There was growth of hair on the face, necessitating shaving once a week. She also complained of frequent headaches and nausea. Little by little she became apathetic and her muscular and intellectual powers declined. Her menstrual periods had stopped two years previously. In April 1956 the positive findings in a local hospital were a Cushingoid habitus, eosinopenia, decreased glucose tolerance and an enlarged sella on x-ray examination. These findings were confirmed in the neurological department of this hospital, and she was transferred to the medical department for further study and treatment.

On admission on May 30, 1956, she felt reasonably well except for asthenia. Her face was round and plethoric with slight hirsutism. She was obese, the obesity being limited to the face, neck, shoulders and abdomen; the extremities were thin. She had many purplish striae over the abdomen, and also ecchymotic areas, particularly over the upper extremities. Her face, chest and abdomen were deeply pigmented, as were the palms of the hands, the knuckles of the fingers, and the elbows. Areolar pigmentation was increased. No oral pigmentation, and no freckles, vitiligo or leukoderma were observed. The blood pressure was 145 mm. Hg systolic and 95 mm. diastolic.

Laboratory data: The hemoglobin level and red and white blood cell counts were within normal limits. There were 19 eosinophils per cu. mm. The oral glucose tolerance test after 50 Gm. of glucose showed the following blood sugar levels: control, 109 mg. per 100 ml.; thirty minutes, 219 mg.; one hour, 224 mg.; two hours, 153 mg.; and three

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hours, 105 mg. There was infrequent spontaneous glycosuria, but no proteinuria. The blood electrolyte levels were within normal limits: total base, 153 mEq. per liter; potassium, 4 mEq.; chlorides, 101 mEq.; and total bicarbonate content, 24 mEq. per liter. The basal metabolic rate was −8 per cent.

X-ray studies showed an enlarged sella turcica with osteoporosis of the dorsum; there was no decalcification of the spine. Perirenal insufflation did not reveal any masses, but the adrenal shadows were reported larger than normal, and the radiologist suggested hyperplasia of the adrenal glands. An electroencephalogram was within normal limits, as were also the results of neurologic examination. Visual fields and funduscopic findings were normal.

The clinical diagnosis of Cushing's syndrome due to a pituitary tumor was made, although the pigmentation was unusual. The patient was given a course of deep x-ray therapy to the pituitary area and was discharged after a six-week stay in the hospital. At home her muscular strength returned, and the facial hirsutism decreased. Her appearance, however, at the time of a control examination four months later was completely unchanged.

On readmission in October 1956, sixteen months after her first stay in the hospital, she complained of fatigue, backache and swollen legs. Her beard had increased in density. The Cushingoid appearance was unchanged, the pigmentation deeper, and there was pitting edema of the legs. There was no change in the sella turcica. Her condition went steadily downhill. The backache (due to osteoporosis) increased. She was discharged on October 31, 1956.

On readmission more than a year later, in November 1957, she was completely bedridden. The pigmentation was still deeper, the ecchymoses larger, and the muscles thin and flabby. It was felt that the patient should be operated on, and on June 6, 1958, craniotomy and hypophysectomy were performed. A small tumor was found upon the diaphragma sellae. Frozen sections showed atypical tumor tissue. The diaphragm was split and the sella emptied. In the neurosurgeon's opinion, however, the hypophysectomy was incomplete.

There was no improvement after operation. On the contrary, the patient's condition deteriorated slowly and she died on October 19, 1958.

Hormone Excretion

The results of hormone studies during her stay in the hospital are listed in Table 1. The excretion of 17-ketosteroids was normal on every examination, with a range between 3.5 and 7.2 mg per twenty-four hours. Upon administration of ACTH (a long-acting preparation, 40 units twice daily for three days) the excretion rose to 21.6 mg. The 17-ketogenic steroid values increased upon administration of ACTH to 108.9 mg. per twenty-four hours, and were thought compatible with hyperplasia of the adrenals. Neither 17-ketosteroid nor the 17-ketogenic steroid excretion changed after operation on the hypophysis.

Autopsy

The autopsy (from which only the positive findings are reported) was performed twenty-four hours post mortem. In the lungs, liver, spleen, kidneys and adrenals, miliary necrotic foci were found surrounded by granulated leukocytes. These foci contained numerous tubercle bacilli. The adrenal glands were enlarged, weighing in all 40 Gm. The enlargement was due to an increased thickness of the zona fasciculata. The cells were irregular with eosinophilic, finely granulated cytoplasm.

The sella was removed and cut frontally. The bony tissue was soft and cut very

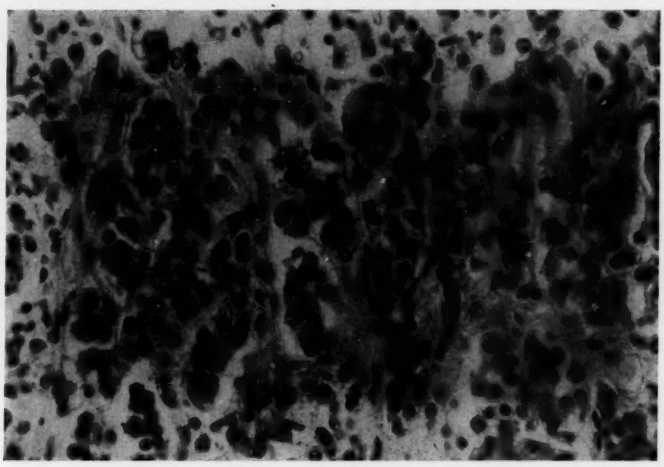

FIG. 1. Section from a nodule of tumor cells, showing a rest of well organized pituitary tissue. Hematoxylin-Eosin ×320.

easily. Sections from this region showed lobulated tumor tissue that filled the cavity and further infiltrated and partly destroyed the sphenoid bone and laterally surrounded the carotid arteries. Microscopically the tumor cells were grouped in irregular nodules, some of which contained highly differentiated, mostly chromophobe cells (Fig. 1); others were made up of interlacing strands and bundles of slightly enlarged and elongated cells with round, even nuclei (Fig. 2). Most of the tumor was, however, composed of irregular polymorphous highly anaplastic cells loosely arranged around tiny vessels with scanty adventitial fibrous tissue (Fig. 3). The cytoplasm in these cells was pale, vacuolated or homogenous; the nuclei were large and lobulated, and a few mitoses were seen. Only a few cells containing PAS-positive granules were found.

Autopsy diagnosis: Carcinoma of the pituitary gland. Bilateral adrenal hyperplasia. Miliary tuberculosis.

DISCUSSION

The frequency of malignant tumors of the pituitary gland varies in different series. Puestow (1) found 8 carcinomas among 50 adenomas, Bailey and Cutler (2) 3 among 88, Thompson and Eisenhardt (3) 1 among 39, and Wise *et al.* (4) 4 among 172 pituitary tumors. They are rare, although according to Jackson (5), they are not as infrequent as commonly assumed. However, the association of malignant tumors of the hypophysis with Cushing's syndrome is extremely rare, and only a few cases have been reported. Bergstrand (6) published a case due to a basophilic adenoma infiltrating the cavernous sinus. Cohen and Dible (7) described a patient with a basophilic adenocarcinoma and metastases to the liver. The metastatic cells were similar to the cells of the pituitary tumor. Metastases to the liver were also found in cases published by Forbes (8) and by Sheldon, Golden

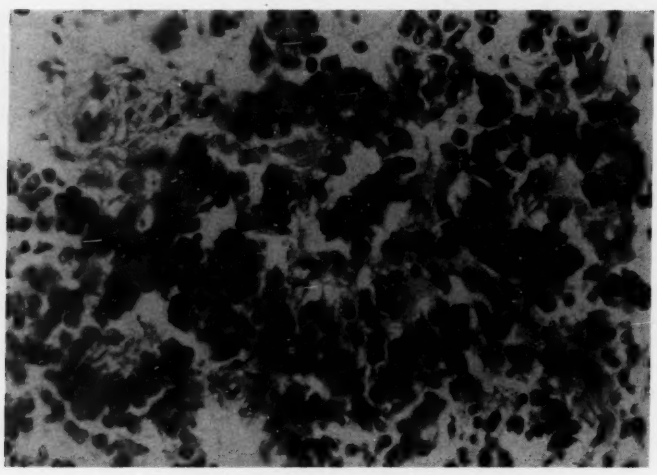

FIG. 2. Interlacing bundles of slightly enlarged and elongated tumor cells. Hematoxylin-Eosin ×320.

and Bondy (9). In Forbes' patient the primary tumor was found to contain predominantly chromophobe cells, whereas the cells of the liver metastases were mostly eosinophilic. Of the 3 primary malignant tumors in Feiring, Davidoff and Zimmerman's series (10), 1 was associated with Cushing's syndrome, as was also the malignant tumor in Thompson and Eisenhardt's

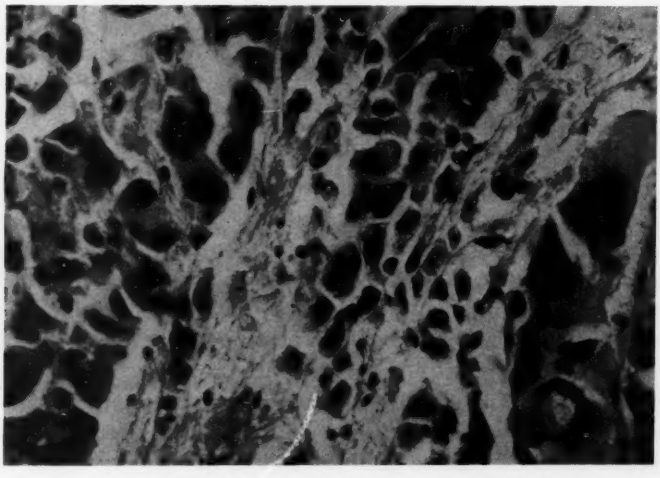

FIG. 3. Highly anaplastic tumor cells. Hematoxylin-Eosin ×320.

(3) series. Basophil adenocarcinomas were described by Siebenmann (11) and by Doret (12).

Cases of Cushing's syndrome due to carcinoma of the pituitary gland should obviously not be treated by surgical procedures upon the adrenal glands, but by hypophysectomy and postoperative irradiation of the pituitary area. The problem is that of a correct preoperative diagnosis. In our opinion the following signs are important:

1) *Enlarged sella due to a large tumor.* In Cushing's syndrome the sella is usually of normal size roentgenologically, and if a pituitary tumor is found

TABLE 1. THE EXCRETION OF 17-KETOSTEROIDS AND 17-KETOGENIC STEROIDS DURING THE PATIENT'S STAY IN HOSPITAL, BEFORE AND AFTER THE ADMINISTRATION OF ACTH

Date	17-KS (mg./24 hrs.)	17-KGS (mg./24 hrs.)
June 6, 1956	3.5	
June 13, 1956	4.6	
June 16, 1956	3.9	
Oct. 8, 1956	6.6	
June 23, 1957	4.9	20.7
June 24, 1957	5.9	15.4
June 25, 1957	7.2	41.5
June 26, 1957	14.3 } Long-acting ACTH,	{ 92.3
June 27, 1957	21.6 } 40 units twice daily	{108.9
June 6, 1958, Hypophysectomy		
Aug. 21, 1958	11.6	51.6

associated with the syndrome it is usually a small tumor not expanding the sella. Size alone should not be considered a criterion of malignancy because benign tumors may attain large proportions, but enlarged sellas should always arouse a suspicion of malignancy.

2) *Lack of effect of irradiation of the pituitary area.* Although irradiation of the pituitary has been abandoned by most endocrinologists, in grave cases of Cushing's syndrome a fair trial seems reasonable when the sella is found enlarged. In spite of some subjective improvement, there was no improvement in our patient's habitus and no decrease of pigmentation. In our opinion, no benefit was obtained from the irradiation. In the cases described by Cohen and Dible (7) and by Siebenmann (11) there was only a transient improvement, and Forbes' (8) patient failed to respond both to irradiation and implantation of radon seeds with the sella. Although lack of effect also may be seen in cases of Cushing's syndrome not associated with

pituitary tumors, failure lends additional weight to the suspicion of malignancy.

3) *Pigmentation of the skin.* This is the most important criterion of malignancy. In Cushing's syndrome there is no distinct pigmentation, but the skin usually assumes a dusky hue, mostly diffuse, and exaggerated on the face and the extremities. In Cushing's syndrome associated with pituitary carcinoma there seems to be a more pronounced pigmentation. In Cohen and Dible's (7) patient there was "brownish pigmentation especially of the face, nipples, axillae, lower abdomen and legs below the knees." The skin of Forbes' (8) patient was "dusky-red," and at autopsy "the entire skin was of a distinct brownish-coppery colour, especially over the upper parts of the body." In Doret's (12) patient "une pigmentation brun-noire" of the wrinkles of the forehead, of an operative scar and of the nipples was observed. However, although deeply pigmented, none of these patients showed a pigmentation comparable to that of our patient. In addition to the dusky hue regularly seen in cases of Cushing's syndrome there was also a pigmentation highly characteristic of Addison's disease, localized to pressure points such as the elbows, the knuckles of the fingers and the palms of the hands. There was also an increase in intensity of areolar pigmentation. The pigmentation was of sufficient magnitude and extension to suggest a diagnosis of Addison's disease to doctors who had not seen the patient before.

In Addison's disease the pigmentation is thought to be due to increased amounts of circulating melanocyte-stimulating hormone (MSH) and adrenocorticotropin (ACTH). The same probably applies to our patient with Cushing's syndrome. In the Addisonian patient the pigmentation decreases upon administration of cortisone by inhibiting the secretion of pituitary MSH and ACTH. In our patient the increased production of glucocorticoid hormones theoretically should have inhibited the secretion of MSH and ACTH. This was not the case, and such a finding suggests that the malignant cells secreted MSH and ACTH, the secretion being of such magnitude that the patient's own glucocorticoid production was unable to cope with it. Secretion of increased amounts of both ACTH and MSH in a patient suffering from a chromophobe adenoma was recently described by Nelson *et al.* (13).

The rather unexpected, though not surprising, finding of generalized tuberculosis needs no further comment, since a lowered resistance to infections in Cushing's syndrome is well known. The generalization of the tuberculosis must have occurred shortly before death, because no signs of the disease had been present in roentgenograms of the lungs.

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URINARY KETOSTEROIDS AND PREGNANETRIOL IN HIRSUTISM

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ABSTRACT

The urinary excretion of 17-ketosteroids and pregnanetriol was compared in a group of normal young women and in a group of 11 women whose presenting complaint was hirsutism. Androsterone, etiocholanolone, dehydroepiandrosterone, 11-hydroxyetiocholanolone, 11-ketoetiocholanolone, and 11-hydroxyandrosterone were determined quantitatively by paper chromatography. Among the hirsute women, an elevated excretion of androsterone and etiocholanolone was noted in 6, and of dehydroepiandrosterone in 5. The 11-oxyketosteroids tended to be higher in the hirsute group, although no statistical significance was attained. Pregnanetriol excretion was normal.

Hirsutism may be associated with elevated excretion of 11-deoxyketosteroids, but it is impossible to predict clinically which patients will fall into this group. There is little overlap clinically between hirsute women and virilized women. This suggests a qualitative difference in androgen production. The significance of the association of elevated excretion of androgen metabolites and hirsutism remains to be determined.

ALTHOUGH hirsutism does not constitute a major medical disability, its association with menstrual disturbances, infertility, and a variety of neoplasms increases its significance. The finding that the excretion of 17-ketosteroids is somewhat elevated in many of these patients has intrigued the endocrinologist and stimulated further inquiry into the identity of these ketosteroids. For these reasons we have examined, in detail, some of the ketosteroid metabolites that may reflect the endogenous secretion of androgenic steroids.

METHODS

Eleven women with hirsutism as one of the presenting complaints were studied. In several instances, hirsutism was only one feature of a clinical problem involving menstrual disturbances and infertility. Salient characteristics of these 11 women are noted in Table 1. None had a masculine habitus, deep voice, balding, or clitoral hypertrophy. The hirsutism was roughly graded from + to +++ according to the extent and distribution of facial and body hair.

Urine was collected in 24-hour specimens, without preservative. The results reported are the average of the analyses of 1 to 3 urine collections for each patient. Appropriate

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aliquots of the urine were incubated for five days with beef-liver β-glucuronidase (Ketodase®). They were then continuously extracted with ether for forty-eight hours at pH 1.0, and for twenty-four hours at 1.0 *N* sulfuric acid concentration. The extracts were washed with alkali and dried under air.

For ketosteroid determinations, aliquots of crude extracts were applied to Whatman 3 MM paper. In each instance one aliquot of the urine was stained on paper and another aliquot was eluted with methanol for analysis by the micro-Zimmermann reaction. The values obtained as dehydroisoandrosterone were converted to milligrams of steroid present (1).

The following abbreviations and trivial names have been used:

D = 5-androsten-3β-ol-17-one (dehydroepiandrosterone),
A = androstan-3α-ol-17-one (androsterone),
E = etiocholan-3α-ol-17-one (etiocholanolone),
OE = etiocholan-3α-ol-11,17-dione,
OHE = etiocholan-3α,11β-diol-17-one,
OHA = androstan-3α,11β-diol-17-one,
Pregnanetriol = pregnan-3α,17α,20α-triol,
17α-Hydroxyprogesterone = 4-pregnen-17α-ol-3,20-dione.

D, A and E were chromatographed in the system iso-octane/methanol:water, 10/8:2, for sixteen hours. Recovery of these steroids added to urine before hydrolysis averaged 81 per cent. OHE, OHA and OE were chromatographed in the system iso-octane: toluene/methanol:water, 3:1/3:1, for twenty-four hours.

In some of the urines examined, a contaminant slightly less polar than OHA that produced an instant brilliant blue reaction on staining the paper with dinitrophenylhydrazine, interfered with the determination of OHA. Subsequent chromatrography in toluene-propylene glycol separated it from OHA.

Pregnanetriol was determined on the foregoing hydrolysates by ascending chromatography using a modification of the Eberlein-Bongiovanni system (2), isooctane:toluene/methanol:water, 20:30/45:5. Allo-pregnanetriol has approximately the same mobility as pregnanetriol in this stystem. Sulfuric-acid chromogens were determined using the Allen correction formula after elution of appropriate areas. Recovery of pregnanetriol added before hydrolysis averaged 83 per cent.

Crude 17-ketosteroids (17-KS) were determined by the method of Werbin and Ong (3), and urinary Silber-Porter chromogens by a modification of their method (4).

RESULTS

Data on the urinary excretion of 17-KS in the hirsute women are recorded in Table 1. In common with others, we found the mean of the 17-KS excretion (12 mg. per twenty-four hours) to be above the mean value for normal women in this laboratory (8.5 mg. per twenty-four hours ($P < .02$)).

The excretions of D, A and E are charted in Figure 1. The values for our normal group were comparable to those of Jailer *et al.* (5) and slightly less than those of Kappas and Gallagher (6). The range of the normal values for D was wide (0.25 to 2.6 mg. per twenty-four hours), so that other statistical parameters would not be informative.

Urinary A and E levels varied considerably among the hirsute women.

TABLE 1. EXCRETION OF 17-KETOSTEROIDS IN 11 HIRSUTE WOMEN

Patient	Age (yrs.)	Age at onset of hirsutism	Degree of hirsutism	Acne	Menstrual history*	Average excretion of 17-KS (mg./24 hrs.)	Remarks
C.L.	25	14–16	+++	0	R	8.4	
E.R.	39	19	+	0	I	12.0	
J.H.	29	20	++	+	R	15.0	
M.K.	37	15–16	+	0	R	17.0	
A.S.	31	19–20	++	0	O	10.0	Obesity; S-L†
S.G.	14	11–12	+++	0	I	11.0	True precocious puberty
Z.M.	25	18–20	+	0	O	13.0	S-L†
S.H.	17	14	++	0	I	11.0	
S.F.	17	15–16	++	+	I	11.0	S-L†
V.B.	27	24–25	+++	0	I	12.0	
D.S.	34	—	++	+	I	10.0	

* R = regular menses; I = irregular menses; O = oligomenorrhea.
† S-L = Stein-Leventhal ovaries (polycystic).

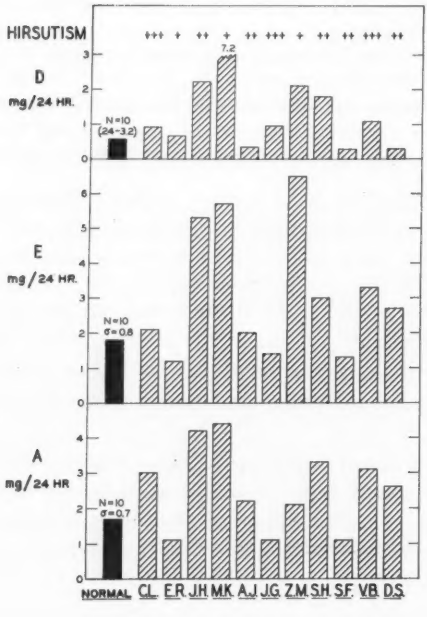

FIG. 1. Excretion of 11-deoxyketosteroids in normal women (black columns) and in each of 11 hirsute women (cross-hatched columns).

Six of them were above the normal range. With only one exception, the ratio of E to A was close to 1.0, the average being 1.3. The E to A ratio of the control group was 1.1, although the range of the ratio was from 0.5 to 2.4. One subject excreted 7 mg. of D, and 4 others had elevated levels.

In Table 2, the data for OHE, OHA, OE and pregnanetriol are listed. The normal values for 11-oxygenated 17-KS are in approximate agreement with those of Kappas and Gallagher (6). Pregnanetriol excretion in our

TABLE 2. COMPARISON OF EXCRETIONS OF OHE, OHA, OE AND PREGNANETRIOL BETWEEN NORMAL AND HIRSUTE WOMEN

	Normal women		Hirsute women	
	Avge. excretion (mg./24 hrs.)	Range	Avge. excretion (mg./24 hrs.)	Range
OHE	.54 [9]*	.30–0.6	.63 [11]	.40–0.9
OHA	.57 [7]	.20–0.8	.70 [9]	.40–1.0
OE	.60 [9]	.30–0.9	.68 [11]	.40–0.8
Pregnanetriol	.87 [11]	.47–1.6	.85 [8]	.45–1.8

* Numbers in brackets indicate the number of patients examined.

control group was slightly below that reported by Stern (7) using column chromatography.

The hirsute women excreted slightly more 11-oxygenated 17-KS than did the control group. OHA showed the largest increment, but even this difference was of questionable significance when tested statistically ($P < 0.2$).

Pregnanetriol excretion was within normal limits in the hirsute group. In all of the subjects, the plasma and urinary levels of Silber-Porter chromogens were normal. In each of 3 patients, suppression of adrenal secretion by one of the derivatives of hydrocortisone was demonstrable.

DISCUSSION

Several questions arise from the results of this and similar investigations: 1) Is there necessarily a correlation between hirsutism and any of the measured steroid metabolites? 2) What is the nature of the abnormality of steroid hormone production? 3) What functional abnormalities would explain these findings?

In each reported detailed study of the urinary ketosteroids in groups of hirsute women, a significant number of subjects have been shown to excrete increased amounts of androsterone and etiocholanolone. Although the percentage of these patients has varied among the groups, the association of hirsutism and elevated levels of A and E is clear in some patients. As

Gallagher *et al.* noted (8), the existence of this relationship does not imply that hirsutism will invariably be accompanied by these aberrations in steroid excretion. In other large series (9–11), there have been many patients with hirsutism and normal excretion of ketosteroid fractions. Even in a large group of hirsute patients reported to show "uniformly elevated" levels of $C_{19}O_2$ ketosteroids (12), occasional patients with severe hirsutism had normal amounts of 11-deoxy- and 11-oxyketosteroids. Only in the group examined by Gallagher *et al.* (8) was there a consistently increased excretion of A and E.

The reasons for these discrepancies among various groups as well as the variations within a group are unknown. There may be a factor of selection operative in some of the studies. Alternatively, there may be at least two different populations of hirsute women, in one of which increased end-organ sensitivity to normal androgen levels accounts for the hirsutism. In the other group, the hirsutism may result from increased secretion of androgens. Less likely possibilities are that hirsutism and disordered adrenal physiology are associated defects; that a steroid not measured in any of these fractions specifically stimulates hair growth; or that there are significant differences in the metabolism of androgenic steroids.

It is impossible to select one among these many hypotheses at present. It is evident, however, that hirsutism and elevated excretion of both C_{11}-deoxyketosteroids and C_{11}-oxyketosteroids are often associated. It is likewise apparent that hirsutism is not necessarily accompanied by these changes in steroid excretion.

It is important to note that none of these patients displayed such features of virilism as male habitus, deepening of the voice, enlargement of the thyroid cartilage, frontal balding, or significant clitoral hypertrophy. In the other reports mentioned, the patients did not have virilism, but rather hirsutism. This could perhaps be used as evidence for the increased production of a substance predominantly affecting the hair follicles. It is more likely, however, that the hair follicle is a more sensitive indicator of low degrees of androgen excess. It is impressive that, on purely clinical grounds, there is little difficulty in classifying patients as examples of either virilism or hirsutism. If there were a continuous range of increasing androgen secretion, one would expect a continuous spectrum of hirsutism merging into virilism. Thus, the aberration of steroid secretion must differ either quantitatively or qualitatively from that in patients with either adrenal tumors or congenital adrenal hyperplasia.

There was no apparent association between the degree of hirsutism and the excretion of androsterone, etiocholanolone, dehydroepiandrosterone, or the sum of androsterone plus etiocholanolone. Two of the patients with the most severe hirsutism had normal ketosteroid patterns, whereas the

largest excretion of 11-deoxyketosteroids was noted in a patient with minimal hirsutism. In the large series of Perloff *et al.* (12), there was likewise a poor correlation. These findings are not particularly surprising, since in view of the multiple factors conditioning the rate of hair growth, a close correspondence between androgens and hair growth would not be expected.

Gallagher *et al.* (8) presented evidence suggesting that the excessive amounts of androsterone and etiocholanolone are a result of adrenocortical rather than ovarian secretion. The elevation of OHA noted by Gallagher *et al.* (8), as well as the suggestion of elevated OHA noted by us and the generally higher $C_{19}O_3$ fractions obtained by Perloff *et al.* (12) further support the view that the adrenal gland in this condition is the site of the increased steroid synthesis. As OHA is the principal metabolite of 11-β-hydroxyandrostenedione (13), these findings indicate an increased production of this weak androgen.

Since pregnanetriol is excreted in large amounts in the adrenogenital syndrome because of the metabolic block in the further hydroxylation of 17α-hydroxyprogesterone, its excretion was examined in this group of hirsute women and was found to be normal.

In view of the normal excretion of Silber-Porter chromogens in our series and the elevated levels of hydrocortisone metabolites found by Gallagher *et al.* (8), an enzymatic block cannot be postulated. Thus, in the non-stimulated state, the adrenal gland in these patients appears to be mildly hyperfunctioning with proportionately greater stimulation of the pathway leading to biosynthesis of the C_{19} steroids. It is unlikely that the androgens are derived from C_{21} precursors, since the metabolism of 17α-hydroxy-20-keto-21-deoxysteroids leads to etiocholanolone:androsterone ratios greatly in excess of unity.

There have been relatively few studies of the responsiveness of these patients to exogenous adrenocorticotropin (ACTH). In the patient studied by Kappas *et al.* (14), administration of ACTH resulted in a proportionately greater increase in hydrocortisone metabolites than in metabolites of the "adrenal androgens."[1] In two studies of the effects of ACTH stimulation in groups of hirsute women, an excessive increase in pregnanediol and pregnanetriol was observed in one (15), and in pregnanetriol in the other (16). It is difficult to explain these findings on the basis of the postulated "overflow" of the metabolites of intermediates in hydrocortisone biosynthesis, since in Cushing's syndrome due to adrenal hyperplasia the excretion of pregnanetriol is not increased (5). It is tempting to speculate that there is a relative rate-limiting step in these patients between 17α-hydroxyprogesterone and hydrocortisone and that, with ACTH stimulation, adre-

[1] A term used to describe the adrenal precursors of androsterone and etiocholanolone.

nal levels of the hydrocortisone precursors are elevated in a new steady state. Whether this defect in any wise contributes to the excess of "adrenal androgens" is only speculative.

Acknowledgments

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SERUM "THYROGLOBULIN" IN THYROIDAL CARCINOMA*

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ABSTRACT

The butanol-insoluble radioiodine (BII^{131}) in the serum of 34 patients given therapeutic doses of radioiodine was analyzed. In addition to the 2 recognized iodoproteins—one associated with the serum albumin (compound X) and one located between the electrophoretically separated alpha globulins (thyroglobulin)—2 additional "thyroglobulins" in the serum were observed. One of these was associated with the $alpha_1$ globulins, the other with the $alpha_2$ globulins. In a series of 24 patients with carcinoma of the thyroid, the albumin type and the alpha-globulin types of radioiodinated serum protein were found with about equal frequency. So far, in our experience, the alpha-globulin BII^{131} has not been detected in any patient not having carcinoma of the thyroid gland. Only the albumin type of radioiodinated protein was found in the serum in 8 patients with exophthalmic goiter, 1 with Hashimoto's disease and 1 with a normal thyroid gland. Unexpectedly, quantitative analysis of the serum BII^{131} revealed that, on the average, as much of these thyroidally derived proteins was found in the serum after tracer doses of I^{131} as after therapeutic doses in the patients with carcinoma. Wide variations from this average were noted in individual cases. The current concept that the concentration of thyroglobulin in the serum is a function of the intensity of radiation to thyroidal tissue is questioned.

IN 1952, Tong and associates (1) reported the presence of a radioiodinated serum protein in rats which were given 1-millicurie doses of I^{131}. Practically simultaneously, Robbins and co-workers (2) published comparable findings in the case of human thyroidal carcinoma treated with heroic doses of I^{131}. Comparable reports also have appeared regarding patients with exophthalmic goiter after therapeutic doses of radioiodine (3–6).

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During recent years, the unusual radioiodinated serum proteins have been characterized to some extent, primarily by Robbins and associates (7–11). These investigators reported that the substances resembling serum thyroglobulin are insoluble in butanol, unlike protein-bound serum thyroxine. Furthermore, there are actually 2 of these serum compounds, one that migrates electrophoretically with the serum alpha globulins and one that migrates with serum albumin. Since the physicochemical properties of the former matched those of the main protein constituent of the thyroid gland, Robbins and colleagues classified the alpha-globulin iodoprotein as "thyroglobulin." The albuminlike iodoprotein differed in salting-out and other characteristics and was labeled "compound X." Tata and associates (12) doubted that "compound X" is actually one of the serum albumins.

It is the prevailing impression that the appearance in serum of thyroidal iodoproteins is the result of radiation injury to the thyroid gland, and that the greater the radiation the more thyroglobulin is to be found in the serum. This concept has been found to be inapplicable in Hashimoto's disease (13), but perhaps some mechanism for autoimmunity releases thyroglobulin in a manner comparable to that of radiation damage (14).

To assess the radioiodinated compounds in serum, we initiated a program of qualitative and quantitative radiochemical analyses in 1952. We now have studied 124 patients with carcinoma of the thyroid, 109 with Graves' disease and 10 euthyroidal patients who were treated for cardiac or pulmonary reasons, as well as a series of patients with Hashimoto's disease; the results in this last series already have been published (13). For the sake of brevity, only a limited number of the more extensively analyzed cases will be presented in detail; this includes 24 patients with thyroidal carcinoma, 8 with primary or recurrent exophthalmic goiter, 1 with coronary heart disease, and 1 with Hashimoto's disease who received 4 millicuries (mc.) of I^{131} preoperatively.

METHODS

Chemical separation of radioiodinated substances in serum: quantitative analysis. The method has been described previously in this journal (13). It consists essentially of assaying the I^{131} of a sample (1 to 6 ml.) of whole serum, that of the protein precipitate from this sample using the Somogyi technic (PBI^{131}), and that of the residue after thorough extraction of the protein precipitate with acidified n-butyl alcohol (the butanol-insoluble radioiodine, or BII^{131}).

The difference between the total serum I^{131} and the PBI^{131} is taken to represent $iodide^{131}$. Chromatography of such material has failed uniformly to reveal thyronines or tyrosines.

The difference between the value for PBI^{131} and that for BII^{131}, which is the butanol-extractable radioiodine (BEI^{131}), represents thyronines (and any tyrosines, were they present), although chromatography of this fraction frequently shows traces of $iodide^{131}$.

The BII^{131} is potentially a mixture of various proteins, namely, "thyroglobulin,"

"compound X" and 2 other compounds to be mentioned later. These proteins obviously are iodinated, since they are detected by their content of I^{131}; their appearance in the blood almost certainly requires thyroidal tissue, for no other tissue is known to be able to convert iodide to organic forms. The descriptive term "serum thyro-iodoprotein" (at times abbreviated to "TG") will be used for the 4 (or more) serum compounds in this category; this term signifies the location in the serum, the origin in the thyroid and the iodoprotein nature of these compounds.

Electrophoretic separation of radioiodinated substances in serum: qualitative analysis. Serum was obtained from patients after the administration of therapeutic doses of I^{131}; 20 microliters of the serum was applied to Whatman 3MM paper strips 3 cm. wide. In more recent years, electrophoresis has been carried out for sixteen hours at room temperature at 5 milliamperes per eight paper strips, employing a standard commercial apparatus of the Durrum type, with 0.05*M* barbiturate buffer at pH 8.6. At the conclusion of each run, the strips were stretched horizontally and allowed to dry spontaneously at room temperature. Each strip was scanned through a slit 1.0, 2.5 or 5.0 mm. wide (depending on the radioactivity) by a Geiger-Müller end-window tube attached to a ratemeter and in turn to a recording device. The strip was then placed in a beaker and completely covered with butyl alcohol saturated with 2*N* hydrochloric acid. After standing overnight, the strip was washed twice with acidified butanol and again dried. The extracted strip was scanned as before, the two tracings from the same strip now being directly comparable. The strip was heated in an oven and was stained with bromophenol blue or amidoschwarz to localize the bands of protein, or it was cut in two, lengthwise, to permit staining half with a protein-staining dye and half by means of the periodic acid-Schiff (PAS) method to locate the glycoproteins. No visible difference could be detected between stained strips that had and had not been extracted with the acidified alcohol.

Quantitation of the recorded zones of radioactivity was carried out by planimetry. If the pattern was too weak for direct evaluation, paired extracted and unextracted strips were taped to no-screen kodak x-ray film and left for about two weeks. The developed film often permitted localization of radioactivity but not quantitation because of the minimal changes usually observed on the film.

Figure 1 represents the albumin type of "TG" in the serum of a patient with carcinoma of the thyroid, one and two days after administration of 150 mc. of I^{131}; in addition to the large albumin radioactivity, a small amount of alpha-globulin "TG" is recognizable. The difference between the unextracted strip (Fig. 1, above) and the extracted strip (Fig. 1, below) indicates the BEI^{131}, primarily thyroxine. Figure 2 is an example of alpha-globulin "TG" after use of 200 mc. of I^{131}; a small albumin "TG" component is visible in all 5 serums.

Justification of the chemical and electrophoretic methods. The addition of radioiodide, radiothyroxine, radiotriiodothyronine and radioiodinated human serum albumin to nonradioactive human serum furnished a simple means of testing for BII^{131} chemically. It was found that 1.8 to 5.4 per cent of $iodide^{131}$ remained with the washed protein precipitate, whereas the corresponding figures were 92 to 96 per cent for radiothyroxine and 95 to 98 per cent for radiotriiodothyronine and radioiodinated albumin. After extraction of the precipitate with acidified butanol, less than 2 per cent of the two thyronines remained, but none of the albumin-I^{131} was removed. A saline extract of a radioactive (I^{131}) thyroid gland, when added to serum, exhibited almost all its radioactivity in the BII^{131}, as would be expected of thyroglobulin.

The electrophoretic identification of serum thyro-iodoprotein was controlled in the same way, namely, the addition of known radioactive substances to nonradioactive human serum. $Iodide^{131}$ migrated off the paper strip completely. Thyroxine-I^{131} migrated

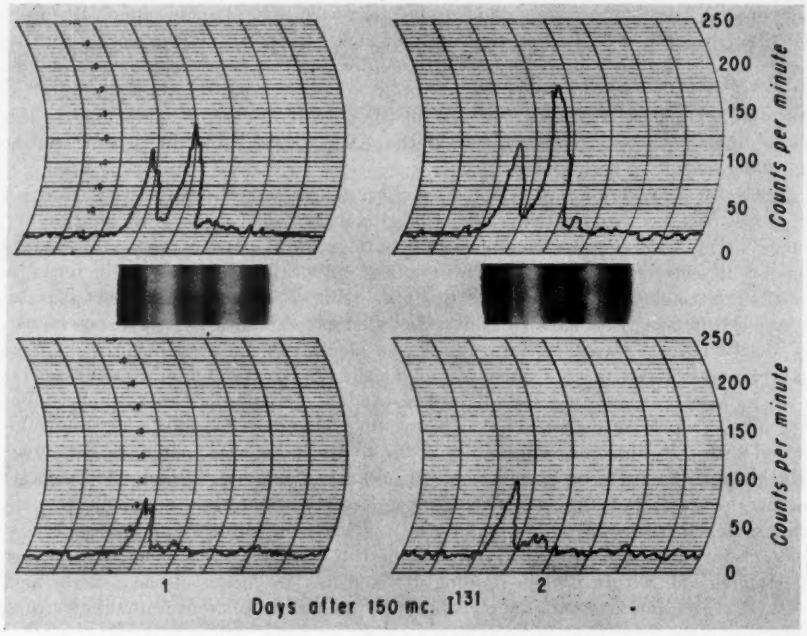
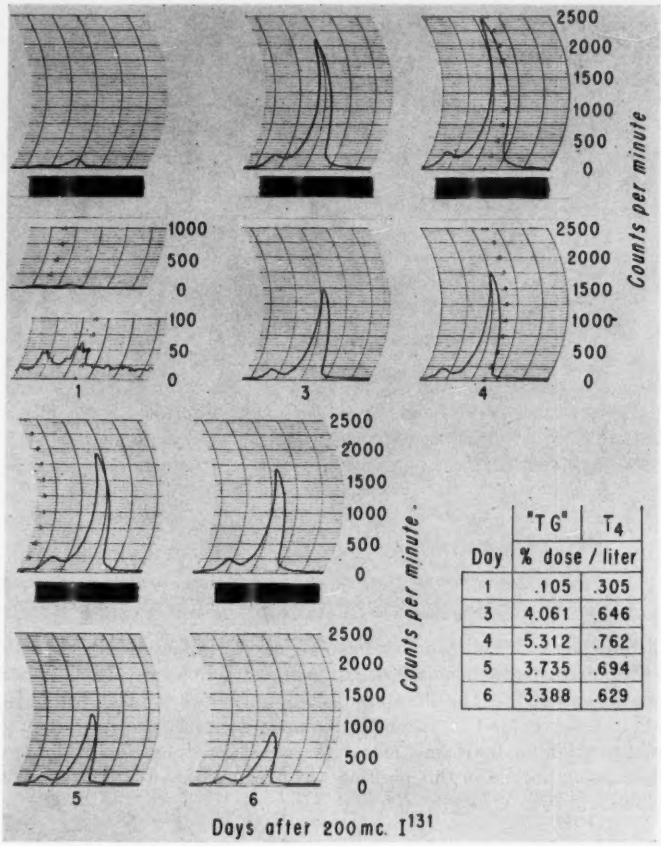


FIG. 1. Most of the I^{131} in the serum in Case 9 (carcinoma of the thyroid) after extraction of the paper electrophoretic strip (lower records) coincides with the albumin bands; therefore, most of the interalpha I^{131} (before extraction, upper records) represents the normal position for thyroxine-I^{131}. The protein bands (left to right) are albumin, $alpha_1$ globulin, $alpha_2$ globulin, beta globulin and gamma globulin.

to the expected location between the alpha globulins (interalpha zone) but was quantitatively removed by the acid-alcohol treatment; even when the thyroxine-binding capacity of the alpha globulins was exceeded, the excess in the albumin area also was quantitatively extractable. A naturally occurring replica of this experiment is seen in Figure 3. The I^{131} in the serum of this patient with Graves' disease was found in its normal interalpha position, plus overflow to the albumin (Fig. 3, above). There was no evidence of residual I^{131} in either zone after extraction of the strip (Fig. 3, below). Radiotriiodothyronine could not be identified in the electrophoretic strips after extraction. On the other hand, radioiodinated serum albumin became localized in the albumin zone and was unaffected by extraction. Similarly, an extract of radioactive thyroid gland, localized mainly with the alpha globulins and to a slight extent with the albumin (Fig. 4), showed no reduction of radioactivity after extraction.

To insure accuracy, all electrophoretic strips were prepared in duplicate, with further replicates if the initial findings were unexpected or inconsistent. A minute amount of "TG" could be identified in serum by the finding of a consistent zone of I^{131} in repeated scans of one strip and in replicate strips.

If the chemical and electrophoretic technics are both valid, their BEI^{131}/PBI^{131} ratios should be identical for each serum. To test this, a random series of 28 studies on serums from patients with hyperthyroidism, Hashimoto's disease or thyroidal carcinoma were compared statistically. In these 28 analyses, the chemical BEI^{131}/PBI^{131} ratio averaged



Day	"TG" % dose / liter	T_4
1	.105	.305
3	4.061	.646
4	5.312	.762
5	3.735	.694
6	3.388	.629

FIG. 2. Series of electrophoretic patterns (upper strip stained with bromophenol blue and lower [faint] strip stained by the PAS method) and radioactive tracings from serum in Case 13 (carcinoma of the thyroid) after a therapeutic dose of I^{131}. Except on the first day, when the values for albumin and alpha "thyroglobulins" were about equal, the alpha type predominated. The concentrations of I^{131} as "TG" (BII^{131}) and "T_4" (thyronine-I^{131}) are tabulated.

71.9 per cent; the electrophoretic value, expressed as radioactivity removed by extraction (comparable to BEI^{131}), divided by the radioactivity on the unextracted strip (PBI^{131}), was 71.0 per cent. Of the 28 pairs of values, 13 agreed within 5 per cent of the smaller value, 24 were within 10 per cent and none showed a difference of more than 20 per cent.

RESULTS

Qualitative analysis of serum "TG"

A summary of the electrophoretic classification of serum thyro-iodoproteins is shown in Table 1. If both the albumin and alpha-globulin varieties

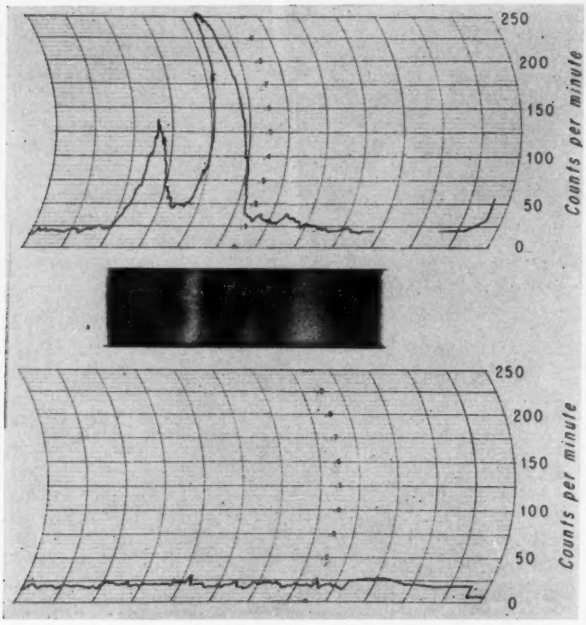


FIG. 3. Tracings of I^{131} in an unextracted paper electrophoretic strip of serum (above) and in a strip extracted with acidified butanol (below) in a case of Graves' disease after a therapeutic dose of I^{131}. The I^{131} appears to be extracted completely and is, therefore, presumably all thyronine-I^{131}. The major component in the unextracted tracing lies in the normal position for thyroxine, namely between the alpha globulins; apparently the thyroxine-binding protein in this position was overtaxed, so that the excess was bound to the albumin.

of "TG" were present in significant amounts, the serum was classified as "mixed."

It can be seen in Table 1 that all the patients with nonmalignant diseases of the thyroid or with a normal thyroid gland (1 patient) exhibited the albumin type of serum "TG." No exception has been found so far in other cases not included in this report. In addition to the one patient with Hashimoto's disease who received 4 mc. of I^{131}, 3 similar patients were studied after large tracer doses, by means of autoradiography; all of them had the albumin type of "TG." Table 1 also shows that 13 of the 24 patients with carcinoma of the thyroid had significant amounts of alpha-globulin "TG" at one time or another. To date, we have found the alpha-globulin "TG" only in patients with thyroidal carcinoma.

Perhaps the finding of alpha-globulin "TG" is simply a function of dose, since most of the patients with carcinoma received the larger doses of I^{131}.

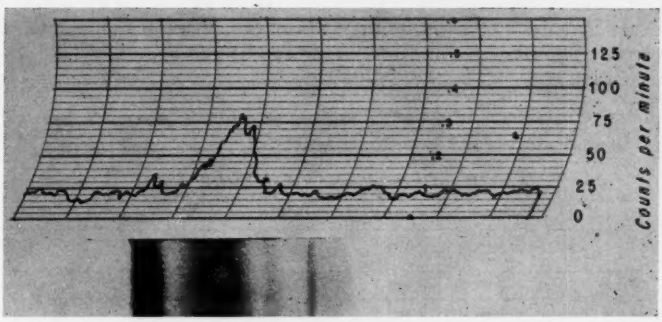


FIG. 4. A mediastinal metastatic lesion of thyroidal carcinoma (Case 14) that contained I^{131} was extracted with a 0.9 per cent solution of sodium chloride. The extract was added in equal volumes to nonradioactive normal human serum. A nonradioactive protein is visible at the point of application of the mixture to the filter paper (at the left edge of the gamma globulin). The main protein constituent of the gland and of the I^{131} from the gland fills the alpha$_1$ and alpha$_2$ zones and obscures the interspace. A small amount of I^{131} coincides with the albumin band.

However, it is to be remembered that the significant factor is not millicuries swallowed but rads per gram of thyroid. A patient with Graves' disease who has an uptake of 80 per cent and a 30-Gm. gland will get more thyroidal irradiation from 5 mc. of I^{131} than will a patient with carcinoma who has an uptake of 10 per cent and metastatic tumors totaling 500 Gm., even if the dose is 200 mc. Since the mass of most metastatic lesions cannot be estimated accurately, attempts at calculating the radiation exposure to the thyroidal tissue were not made.

A summary of the 24 patients with thyroidal carcinoma is presented in

TABLE 1. CLASSIFICATION OF SERUM THYRO-IODOPROTEIN ("TG") IN THYROIDAL CARCINOMA, NORMAL THYROID, HYPERTHYROIDISM AND HASHIMOTO'S DISEASE

No. of patients	Dose of I^{131} (mc.)	Diagnosis	Type of serum "TG"
11	60–202	Carcinoma of thyroid	Albumin
4	193–200	Carcinoma of thyroid	Alpha
6	50–200	Carcinoma of thyroid	Mixed
1	Multiple	Carcinoma of thyroid	Albumin, mixed
2	Multiple	Carcinoma of thyroid	Mixed, albumin
1	30	Coronary heart disease	Albumin
7	4.4–14.7	Exophthalmic goiter	Albumin
1	25	Recurrent Graves' disease	Albumin
1	4	Hashimoto's disease	Albumin

TABLE 2. CLASSIFICATION OF PATIENTS WITH CARCINOMA OF THE THYROID ACCORDING TO TYPE OF SERUM "THYROGLOBULIN"

Patient			Dose of I^{131} (mc.)	Pathologic diagnosis*	Grade of malignancy	Metastasis	Previous I^{131} therapy	Previous thyroid operation
Case No.	Age	Sex						
Predominantly albumin type of "thyroglobulin"								
1	11	M	125	P and F	1	Cerv. nodes	None	Yes†
2	20	M	150 150	P and F	1	Cerv. nodes, trachea	None	Yes†
3	26	F	200	P and F	1	Pulmonary	Yes	None
4‡	27	M	100	P and F	1	None	None	1 yr
5	28	M	200	F	1	Pulmonary	None	Yes†
6	30	F	100 175	P and F	1	Pulmonary	Yes	4 yrs.
7‡	37	M	200	F (HC changes)	1	Cerv. nodes	None	Yes†
8	45	F	100	P and F	1	Cerv. nodes	None	Yes†
9	54	F	150	F	1	Intracranial	Yes	4 mos.
10	59	F	60	F in struma ovarii	1	Pelvic	None	Yes†
11	63	F	202	F and S	2	Pulmonary, skeletal	None	Yes†
Predominantly alpha type of "thyroglobulin"								
12§	37	F	200	F	1	None	None	Yes†
13‖	44	F	200	F	2	Skeletal	None	1 yr.
14¶	61	F	200	F	1	Pulmonary	None	4 yrs.
15	62	M	193	F and S	1	Skeletal	None	9 yrs.
16	50	M	200	F	1	Skeletal	None	Yes†
17	62	F	200	P and F	1	Cerv. nodes	None	4 mos.
18	63	F	150	F	2	Orbit	None	4 yrs.
19	63	M	50	F	2	None	None	Yes†
20**	64	M	200	F and S	2	Cerv. nodes	Yes	3 yrs.
21	68	F	200	F and S	2	Liver	Yes	4 yrs.
Albumin type of "thyroglobulin" initially; mixed types subsequently								
22	65	F	200 (alb.) 144 200 (mixed)	F and S	1	Skeletal	None	3 yrs.
Mixed types of "thyroglobulin" initially; albumin type subsequently								
23§	55	M	185 (mixed) 200 (alb.)	P and F	1	Pulmonary	None	Yes†
24	64	F	180 206 (mixed) 200, 150 194 (alb.)	F	1	Skeletal	Yes	1 yr.

* Pathologic types of malignant tumor: P = papillary; F = follicular; S = solid; HC = Hürthle-cell.
† Operation on thyroid just before the therapeutic dose of I^{131}.
‡ Alpha type of "thyroglobulin" present, but less than half; others of this group had only "trace" amounts.
§ Probably alpha$_2$ type of "thyroglobulin."
‖ Probably alpha$_1$ type of "thyroglobulin."
¶ Alpha$_1$ type of "thyroglobulin."
** Alpha component was of the alpha$_2$ type of "thyroglobulin."

When multiple doses are listed, the type of "thyroglobulin" was identified in each one (except Cases 22 and 24).

Table 2. Slight differences apparently exist between the "TG" groups. Thus, the younger patients tended more toward the albumin type and also had a preponderance of grade 1 carcinoma. The patients with alpha-globulin "TG" (alone or mixed) were somewhat older and had a greater number of grade 2 carcinomas. Sex, duration of illness and the presence of metastasis apparently furnish no clue as to the difference between the types of

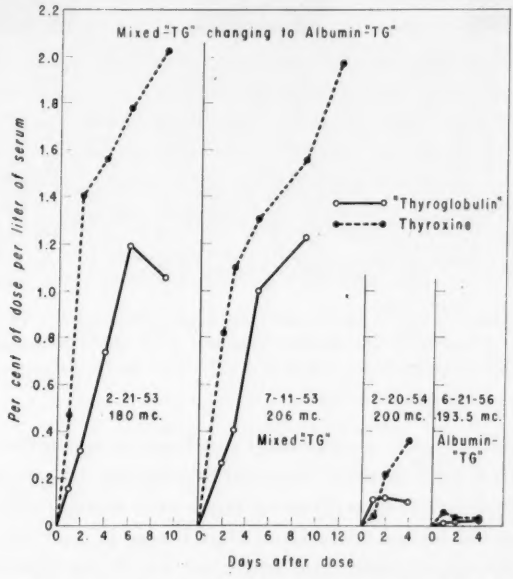


FIG. 5. Qualitative and quantitative changes in the serum "TG" associated with repeated therapeutic doses of I^{131} in Case 24. The "thyroglobulins" were of the alpha-globulin and albumin types after the second treatment, but were almost entirely of the albumin type after the fourth dose of I^{131}.

iodoproteins. Details concerning a patient (Case 24) whose serum contained mixed "TG" on first examination, with change to the albumin type of "TG" later, are shown in Figure 5.

The complexity of the problem was not lessened by the finding in 2 patients (Cases 14 and 20) of alpha-globulin "TG" that appeared to differ from the usual serum "thyroglobulin." In its classic form, the peak activity of alpha "TG" lies approximately midway between the $alpha_1$ and $alpha_2$ globulins of the serum (Fig. 2). In the newly discovered variants, the I^{131} coincided with the $alpha_1$ globulins in one instance (Case 14, Fig. 6) and with the $alpha_2$ globulins in the other (Case 20, Fig. 7). One might suspect

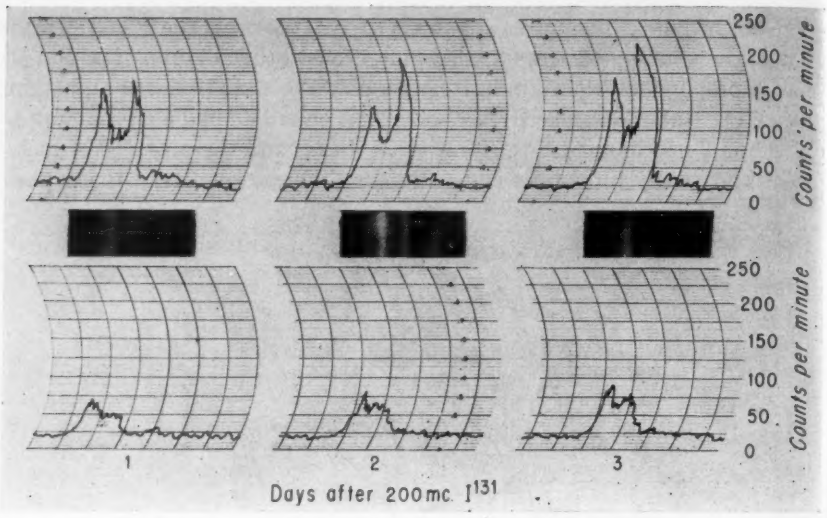


FIG. 6. Distribution of I^{131} among the serum proteins in Case 14 (carcinoma of the thyroid). The tracings from the strips extracted with acidified butanol (below) show both albumin and alpha-globulin types of "TG"; the peak of the globulin-I^{131} tends to coincide with the $alpha_1$-globulin band.

the presence of artifacts, except that duplicate electrophoretic strips were run on each of 3 or 4 serums collected on different days; the results were completely consistent. Once these variants were recognized, re-examination of earlier data led to the realization that Cases 12 and 23 were also probably of the $alpha_2$-globulin type and Case 13 of the $alpha_1$-globulin type. These cases have not been reclassified because the recognition was retrospective and there was no opportunity for the exhaustive checking carried out in Cases 14 and 20.

Case 14: $alpha_1$-globulin type of "TG." The patient was a 61-year-old woman. In 1954 the thyroid had been surgically removed elsewhere and reported to contain cancer. Examination at the Mayo Clinic in 1958 disclosed a large mediastinal mass that proved to be grade 1 follicular carcinoma of the thyroid. After operation, a 200-mc. dose of I^{131} was administered orally; surveys of the body revealed uptake in the thyroidal region of the neck, plus unsuspected uptakes in the lower thoracic vertebrae and sacroiliac region.

Case 20: $alpha_2$-globulin type of "TG." A 64-year-old man had undergone previous thyroidectomy elsewhere, the diagnosis of cancer being made. When he visited the clinic, in 1955, total right and left thyroidectomy was performed, along with radical dissection of the neck on the right. The pathologic diagnosis was grade 2 follicular and solid carcinoma of the thyroid. Four therapeutic doses of I^{131} preceded the present study; these included 100 and 161 mc. in 1955, 200 mc. in 1956, and 200 mc. in 1957. In 1958, after a six-day series of injections of thyroid-stimulating hormone, 200 mc. of

I^{131} was given. Serums obtained after this dose showed an albumin and an alpha$_2$-globulin pattern of "TG."

Hydrolytic products of serum "TG"

The distribution of radioiodinated amino acids in the hydrolytic product of thyroid glands of normal persons (15) and of patients with Hashimoto's disease (13) has been characterized. These values are compared in Table 3 with the distribution of these amino acids in 2 metastatic masses of thyroidal carcinoma and in the butanol-insoluble residue of the serum of 8 patients with thyroidal carcinoma and 1 with Hashimoto's disease.

It appears that the single specimen of serum "TG" from the patient with Hashimoto's disease and the serums from 5 of the 8 patients with carcinoma contained radioiodinated amino acids comparable to those of thyroidal thyroglobulin. The remaining 3 patients (Cases 3, 6 and 15) had only mi-

TABLE 3. RADIOIODINATED AMINO ACIDS RESULTING FROM TRYPSIN HYDROLYSIS OF THYROID AND OF SERUM PROTEIN INSOLUBLE IN ACID-BUTANOL*

Source of hydrolyzed material		Type of serum "TG"	Days after dose of I^{131}	Per cent of total I^{131} in amino acids			
				Monoiodotyrosine-I^{131}	Diiodotyrosine-I^{131}	Thyroxine-I^{131}	Triiodothyronine-I^{131}
Hydrolysates of material from thyroid glands							
Normal thyroid (9 cases)		—	Variable	35.0	46.6	17.2	1.2
Hashimoto's disease (10 cases)		—	Variable	41.0	38.1	19.9	1.0
Metastasis (Case 14)		Alpha globulin	5	55.3	35.6	9.1	0
Metastasis (Case 18)		Mixed	4	51.2	31.3	16.4	1.1
Hydrolysates of butanol-insoluble residue of serum (pooled specimens)							
Hashimoto's disease		Albumin	—	76.6	8.3	15.1	0
Carcinoma	Case 1	Albumin	—	77.2	17.5	5.3	0
	Case 3	Albumin	—	98.6		1.4	Trace
	Case 6	Albumin	—	83.9	16.1	Trace	0
	Case 11	Albumin	—	87.0		13.0	0
	Case 13	Alpha globulin	—	87.8	5.6	6.6	0
	Case 15	Alpha globulin	—	76.8	20.5	2.2	0.5
	Case 18	Mixed	—	78.4	12.1	8.5	1.0
	Case 20	Mixed	—	60.8	23.7	14.5	1.0

* Amino acids were separated by kieselguhr column chromatography; quantitation was by planimetry of the recorded radioactivity.

nute amounts of thyroxine-I^{131} in their butanol-insoluble serum residues; this was unlike thyroglobulin, but very similar to the finding in artificially radioiodinated proteins. For example, 3 proteins, labeled *in vitro* with I^{131} at Abbott Laboratories,[1] were hydrolyzed; human serum albumin yielded 3.7 per cent of its I^{131}-amino acids as thyroxine, whereas the value for human gamma globulin was 0.9 per cent and that for insulin was 3.0 per cent. One might think that the metabolic pathway of iodine in the gland is altered in certain instances of carcinoma of the thyroid. Thus, the first step (trapping of iodide) and the second step (oxidation of iodide) appear to be

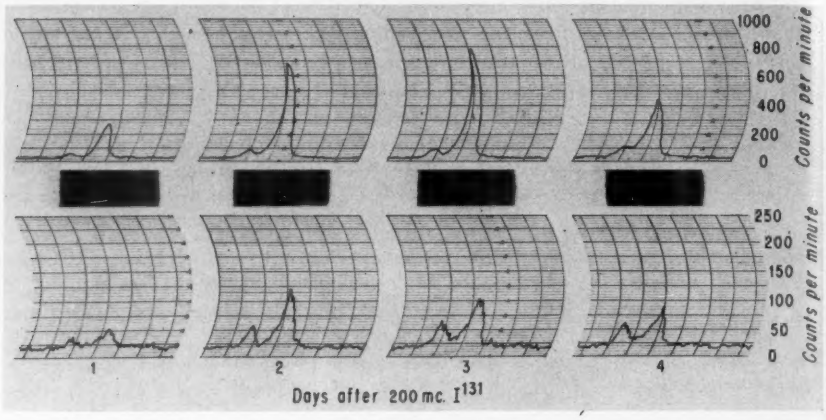


FIG. 7. Distribution of I^{131} among the electrophoretically separated serum proteins in Case 20 (carcinoma of the thyroid), one, two, three and four days after a 200-mc. dose of I^{131}. The radioactive tracings from the extracted paper strips (lower records) show both albumin and alpha-globulin "TG," with the latter predominating; the peak of the alpha I^{131} more closely corresponds with the alpha$_2$ band than with the more usual interalpha zone.

retained, but the normal synthesis of thyroxine, presumably enzymatic, may be replaced by nonenzymatic and nonspecific protein-binding such as occurs with *in vitro* labeling.

Quantitative analysis of serum "TG"

To 13 euthyroidal patients, in whom the presence of carcinoma was suspected but not confirmed at operation, tracer doses of 300 to 500 microcuries of I^{131} were administered. The average concentrations of BII^{131} and BEI^{131} were 0.016 and 0.061, respectively, expressed as a percentage of the dose per liter of serum twenty-four hours after the tracer dose was given. Thus, about one fifth of the protein-precipitable I^{131} was not extractable

[1] Obtained through the courtesy of Dr. Donalee Tabern.

with acidified butanol, which is a figure greater than that obtained when radiothyroxine is added to serum and is similarly treated. In 5 euthyroidal patients after therapeutic doses of I^{131}, the values for BII^{131} and BEI^{131} were virtually the same as those just stated, namely 0.011 and 0.045 per cent of the dose per liter. The apparent presence of "TG" after tracer doses, and in a concentration equal to that of "TG" after therapeutic doses, was unexpected.

A similar comparison was made of all patients with thyroidal carcinoma on whom exactly paired values between the tracer dose and the therapeutic

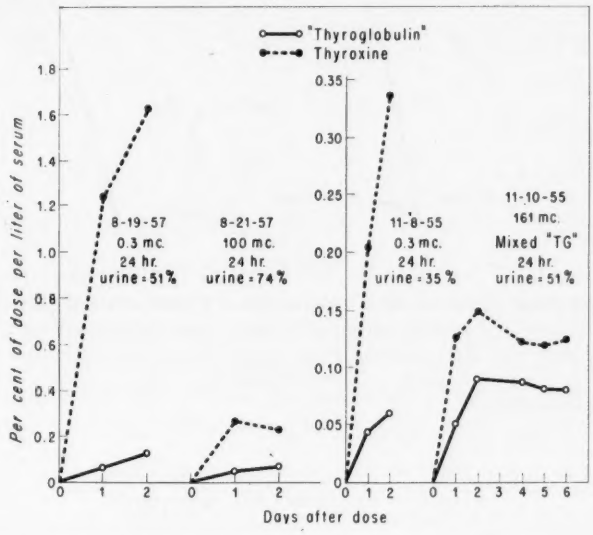


FIG. 8. Comparison of the effect of tracer doses of I^{131} with that of therapeutic doses. The suppression of thyronine-I^{131} in the serum after the therapeutic doses is seen in both cases.

dose were available; 94 paired values were found. After the tracer dose (usually 300 or 500 microcuries of I^{131}), the BII^{131} averaged 0.095 per cent of the dose per liter of serum and the BEI^{131} averaged 0.185 per cent. After the therapeutic dose, the average for BII^{131} was 0.099 per cent and that for BEI^{131} was 0.087 per cent. The significant change was not an increase in the BII^{131} but a reduction in the BEI^{131}. Wide individual variations occurred; 4 examples are shown in Figures 8 and 9.

Of the 94 pairs of values in cancerous patients, 57 pairs were obtained from patients whose serum "TG" had been characterized electrophoretically. A comparison of the results is listed in Table 4. The steadily increasing concentration of the albumin type of "TG" might mean greater radiation

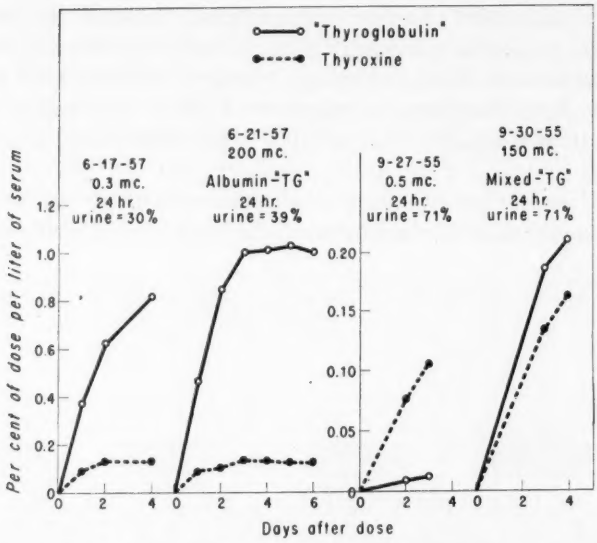


FIG. 9. Comparison of the effect of tracer doses of I^{131} with that of therapeutic doses. In Case 3 (left panel), the concentration of either "thyroglobulin"-I^{131} or thyronine-I^{131} after a tracer dose was little different from that after a therapeutic dose. In Case 18 (right panel), however, a much greater concentration of "thyroglobulin"-I^{131} developed after the therapeutic dose.

TABLE 4. SERUM BII^{131} IN 57 PAIRED TRACER AND THERAPEUTIC VALUES WITH TYPE OF SERUM "TG" IDENTIFIED

Days after dose of I^{131}	Observations	Per cent of dose per liter of serum	
		After tracer dose	After therapeutic dose
Albumin type of serum "TG"			
1	23	0.102	0.087
2	11	0.162	0.198
3–8	7	0.253	0.312
Alpha-globulin type of serum "TG"			
1	8	0.042	0.065
2	5	0.065	0.071
3–8	3	0.058	0.142

TABLE 5. URINARY EXCRETION OF I^{131} AFTER TRACER AND THERAPEUTIC DOSES OF I^{131} IN PATIENTS WITH ALBUMIN "TG" AND THOSE WITH ALPHA-GLOBULIN OR MIXED "TG"

Days after dose of I^{131}	Observations	Cumulative excretion of I^{131} in urine (% of dose)	
		After tracer dose	After therapeutic dose
Albumin type of serum "TG"			
1	22	52.0	46.5
2	10	57.4	56.5
3	3	57.3	58.3
Alpha-globulin type of serum "TG"			
1	8	38.3	45.9
2	5	46.2	63.2
3	3	49.7	75.3

damage, with continuing release of the albuminlike iodoprotein, or one might postulate greater radiation damage in the case of the alpha-globulin "TG," because the synthesis of new thyroglobulin appeared to be depressed during the first day. In either case, the difference in concentrations after diagnostic and therapeutic doses was not as great as one would expect if the concentration in the blood is directly proportional to the radiation to the thyroidal cells.

Since the uptake of I^{131} by metastatic masses cannot be quantitated, determination of the fraction of a dose not excreted in the urine is the only way to estimate uptake. When the urinary excretion of I^{131} of patients with the albumin and the alpha-globulin types of "TG" was compared (Table 5), an apparently significant difference emerged. Despite the limited data, it is apparent that more I^{131} was excreted after the therapeutic dose only in patients who had alpha-globulin "TG" in their serum; this could be explained by increased radiosensitivity of their thyroidal tissue.

Stable BII. It is conceivable, through some radical short-circuiting in the thyroid gland, that the values for radioiodinated protein in the serum might bear little resemblance to those for comparable stable iodinated proteins.[2] The finding that the values for stable iodine paralleled those for radioiodine lends support to the significance of the radioiodine observations. In Table 6 are listed the values for serum I^{131} and I^{127} in 4 patients with carcinoma of the thyroid.

[2] The determinations of stable PBI and BEI were performed by the Section of Biochemistry.

TABLE 6. COMPARISON OF SELECTED VALUES FOR STABLE BII* AND BEI WITH VALUES FOR PBI^{131} AND BEI^{131} IN SERUM IN CARCINOMA OF THYROID

Case No.	Dose of I^{131} (mc.)	Days after dose	Per cent of dose per liter		Micrograms per 100 ml.		Type of serum "TG"
			BII^{131}	BEI^{131}	BII	BEI	
3	0.3	1	0.375	0.091	1.6	2.7	Albumin
		2	0.627	0.131	3.5	2.0	
		4	0.818	0.132	4.7	2.2	
13	200	1	0.105	0.305	0.4	5.0	Alpha globulin
		3	4.061	0.646	14.9	6.1	
		4	5.312	0.762	21.6	6.4	
		5	3.735	0.694	14.5	5.2	
		6	3.388	0.629	11.8	5.4	
14	200	1	0.027	0.101	1.2	3.8	Alpha globulin
		2	0.081	0.253	2.2	4.1	
		3	0.093	0.332	2.6	4.8	
		4	0.103	0.338	3.3	4.8	
20	200	1	0.051	0.056	0.0	1.9	Mixed
		2	0.063	0.064	0.6	1.2	
		3	0.075	0.075	0.4	1.2	
		4	0.068	0.074	0.6	1.0	

* PBI and BEI actually were measured; BII is the difference.

COMMENT

Two fundamental observations were derived from this study; neither was expected and neither is explained.

First, thyroglobulinlike radioiodinated proteins were found in the serum of patients with thyroidal carcinoma whether the doses of I^{131} were "tracer" in amount (up to 0.5 mc.) or at therapeutic levels (up to about 200 mc.). Furthermore, the concentration of this butanol-insoluble serum I^{131} was essentially the same, on the average, within these extremes of dosage. Unless one postulates a remarkable radiation effect from the tracer doses, it is difficult to reconcile these results with the usual view that the concentration of serum thyroglobulin varies directly with the intensity of radiation to thyroidal tissue. Actually, these findings would appear to parallel more closely those of Hashimoto's disease (13).

Secondly, without exception, only the albumin type of serum "TG" was found in various states of the thyroid gland other than carcinoma, and the only patients with the alpha-globulin type of "TG" in the serum were those

who had carcinoma of the thyroid. If the distinction ended here, a diagnostic test for thyroidal carcinoma might be feasible. However, more than half of our series of patients with carcinoma also had the albumin type of serum "TG." Attempts to discriminate between those patients with carcinoma who had one or the other variety of "TG" were not rewarding, although the younger patients tended toward the albumin type of "TG," and the more undifferentiated the tumor the more was the tendency toward alpha-globulin "TG."

It was found also that several varieties of alpha-globulin "TG" may exist in the serum, namely, one corresponding to the $alpha_1$ globulins spatially, one to the zone between the alpha globulins and one to the $alpha_2$-globulin zone. Possibly there is a spectrum of thyroidal iodoproteins that corresponds coincidentally with the various electrophoretic zones of serum protein.

This raises the question as to the origin of the albumin "TG" of the serum ("compound X"). On logical grounds, by exclusion, the thyroid gland necessarily appears to be implicated. It is the one organ known to have the ability to raise iodide to the oxidized state necessary to combine with protein. When the albumin "TG" of serum was hydrolyzed, the radioactive amino acids quantitatively and qualitatively resembled those obtained from extracts of thyroid glands, at least in 3 of 5 instances. That iodinated "albumin" exists in the thyroid gland was recognized sixty years ago (16), but this observation has been generally discounted in recent years. Because of the abnormal distribution of proteins in the thyroid undergoing malignant change (17), perhaps iodoalbumins occur to a greater extent than is commonly thought.

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PROBABLE GENETIC BASIS FOR ABNORMAL CIRCULATING IODOPROTEINS (BUTANOL-INSOLUBLE SERUM IODINE): STUDY OF A FAMILY WITH SEVERAL HYPOTHYROID MEMBERS WITH AND WITHOUT GOITER*

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ABSTRACT

Unusual quantities of circulating iodoproteins, presumably peptide-linked iodo-amino acids, were identified in a 6-year-old hypothyroid boy with goiter, a 2-year-old hypothyroid sister without goiter, and a 59-year-old cousin who was a goitrous cretin. No autoantibodies were identified. The mother, father, a brother and both grandmothers showed no excess of iodoprotein. Chromatographic studies were conducted of the distributions of the iodinated amino acids in the butanol-soluble fractions of all subjects, in hydrolysates of the iodoproteins, and in the digest of the thyroid gland from the 6-year-old boy. The variations from normal are tabulated. The likelihood of abnormal circulating iodoproteins being due to a genetic defect in the thyroid is discussed. Case reports and a pathologic description of the goiter studied chemically are presented.

IODOPROTEINS can be defined as those proteins which contain iodinated amino acids in polypeptide linkage, in contradistinction to carrier proteins upon which the iodinated compounds are adsorbed (1). The iodocompounds of the latter group are readily released upon butanol extraction, whereas those of the former group remain in the insoluble fraction. Until recently, circulating iodoproteins were thought to occur only in association with injury to the thyroid, such as from thyroiditis (2), surgery (3), I^{131} therapy (4), or thyroid cancer (5).

In 1956, it was demonstrated in our laboratories that circulating iodoproteins were present in a cretinous child reported by Whitelaw and collaborators (6). A year later, the same abnormality was observed in a hypothyroid adult with primary amenorrhea (7). Neither of these patients was

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goitrous and neither had had known thyroiditis. The circulating iodoproteins in the serum of the amenorrheic patient were presumably not normal thyroglobulins, as shown by immunologic investigation and by distribution of iodotyrosines and iodothyronines liberated by enzymatic hydrolysis (7).

Shortly thereafter, a markedly hypothyroid boy with a goiter was observed and found also to have iodoproteins in the serum. A younger sister without goiter was said to exhibit the retarded growth pattern of her brother, and a distant cousin was known to be a goitrous cretin. Thus, a study was begun of the serum of several members of the family in order to investigate the possibility that the protein abnormality might have a genetic basis. The results obtained support this concept and indicate that the presence of goiter is not an essential part of the syndrome.

Circulating iodoproteins in a goitrous patient have been identified by DiGeorge and Paschkis (8) and by DeGroot and collaborators (9). The latter investigators showed that the iodoproteins in their patient were present in the thyroid gland as well as in the circulation, and also considered them to represent abnormal polypeptides.

METHODS

The family members studied were: the original patient (J.T.) aged $6\frac{1}{2}$ years, a younger brother (H.T.) aged 4 years, a sister (S.T.) aged $2\frac{1}{3}$ years, the father (E.T.) aged 39, the mother (P.T.) aged 31, both grandmothers (A.T. and H.B.) aged 66 and 59 respectively, and the goitrous cretinous cousin (S.R.) aged 56. The goitrous cousin was located at Letchworth Village, a New York State institution for the feeble-minded, where he had spent most of his life. He was studied through the kind cooperation of Dr. George Jervis. Labeling and chromatographic separation of the iodinated compounds in serum was accomplished by the method of Block, Werner and Mandl (10). A labeling dose of 125 μc. was administered seventy-two hours prior to the drawing of blood. The blood samples were processed the same day, or the following morning after refrigeration overnight. Protein-bound iodine determinations were performed by a modification of the original method of Barker (11); in one instance the determinations were made at Bio-Science Laboratories, Los Angeles, California.

The case reports on Patients J.T., S.T. and S.R. appear at the end of this article.

RESULTS

Forty-three per cent of the radioactivity in the serum of J.T., the index patient, was identified as iodoproteins present in the butanol-insoluble (P-1) fraction (Table 1). In the serum of the sister (S.T.) who was also hypothyroid but without goiter, 39 per cent of the activity was in the same form, whereas in the younger brother (H.T.) only 2 per cent of the activity was in this fraction. The goitrous cretinous cousin (S.R.) with a long-standing huge nodular goiter showed a slight but probably significant elevation of radioactivity in the iodoprotein fraction—24 per cent. The highest normal value obtained with the method is 20 per cent.

TABLE 1. DISTRIBUTION OF THE LABELED IODINATED COMPOUNDS OF SERUM ACCORDING TO SOLUBILITY IN BUTANOL

Subject	Date	Clinical status	Sex	Relative percentage distribution in fractions		
				P-1*	P-2 & 3*	F-1**
J.T.	Dec. 6, 1957	Hypothyroid; goitrous	M	43	7	49
	Apr. 8, 1958			55	3	41
S.T.	Jan. 13, 1958	Hypothyroid	F	38	6	56
H.T.	Feb. 3, 1958	Euthyroid	M	2	27	71
E.T.	Feb. 14, 1958	Euthyroid	M	19	7	74
P.T.	Mar. 10, 1958	Euthyroid	F	5	9	86
A.T.	May 8, 1958	Euthyroid	F	++‡	+‡	+++‡
H.B.	May 8, 1958	Euthyroid	F	7	12	81
S.R.	May 12, 1958	Goitrous cretin	M	24		66

* P-1 = presumably peptide-linked iodoamino acids (iodoproteins), insoluble in butanol; P-2 and 3 = material insoluble in anhydrous butanol.

** F-1 = butanol-soluble iodoamino acids and inorganic iodide (9).

‡ These values inaccurate because of the very weak activity of the serum.

Table 2 shows the distribution of iodoamino acids in the hydrolysate (pancreatin digestion) of the P-1 fraction of serum from Patient J.T. More than half of the original activity, 62 per cent, was found in the form of monoiodotyrosine (MIT) and diiodotyrosine (DIT), but 27 per cent was present as thyronines. The activity was too low in the P-1 fraction of the sister's serum to permit this kind of evaluation.

Study of the iodinated compounds of the serum in the butanol-extractable (F-1) fraction (Table 2) revealed a relatively large amount of I^{131} as iodide in both Patients J.T. and S.R. The traces of DIT plus MIT present in most of the sera were not significant, in view of the weak activity. However, the higher amounts in the patient's brother (H.T.) and sister (S.T.) appeared to be beyond the range of counting error.

The chemical findings upon enzymatic digestion of J.T.'s thyroid gland with pancreatin are shown in Table 3.

Sera from Patients J.T. and S.T. were sent to Dr. E. Witebsky who generously tested them for autoantibodies to thyroglobulin, using the tanned red-cell agglutination method. The results were negative. This finding was confirmed in Patient J.T. by pathologic study of the goiter removed at operation: no evidence of chronic thyroiditis was found. The details of this examination as well as brief case histories of this patient and of his sister, brother and cousin are presented subsequently.

TABLE 2. DISTRIBUTION OF THE LABELED IODINATED COMPOUNDS IN THE BUTANOL-SOLUBLE F-1 FRACTION AND IN THE IODOPROTEIN P-1 FRACTION (4) OF THE SERUM SEVENTY-TWO HOURS AFTER ADMINISTRATION OF I^{131}

Subject	Date	Relative percentage distribution of compounds					
		MIT	DIT	I^-	T_4	T_3	Unknown
J.T.	Dec. 6, 1957	—	2	60	22	15	DIH 1%
	Apr. 8, 1958*	3	5	23	29	39	
	Apr. 8, 1958	11†	15†	18†	39†	18†	
S.T.	Jan. 13, 1958	5	22	34	48	8	
H.T.	Feb. 3, 1958	14	22	30	22	6	Origin 6%
E.T.	Feb. 14, 1958	5	8	36	30	5	Origin 8%; DIH 8%
P.T.	Mar. 10, 1958	5	4	22	33	36	
	Mar. 12, 1958‡	1	2	26	34	37	
A.T.	May 8, 1958	—	1§	48§	50§	1§	
H.B.	May 8, 1958	1	1	16	55	27	
S.R.	May 12, 1958	2	2	60	21	15	
		31†	43†	15†	8†	3†	
J.T. (P-1 hydrolysate)	Dec. 6, 1957	15	47	11	14	13	
S.R. (P-1 hydrolysate)	May 12, 1958	22†	57†	15†	6†	1†	

MIT = monoiodotyrosine; DIT = diiodotyrosine; I^- = stable iodide; T_4 = thyroxine; T_3 = triiodothyronine.

* Ninety-six hours after administration of I^{131}.

† Distribution of I^{127}.

‡ Twenty-four hours after 10 units of TSH.

§ These values are inaccurate because of the very weak activity of the serum (*cf.* ref. 12).

DISCUSSION

The presence of circulating iodoproteins has been established in a hypothyroid boy with goiter (J.T.) and in his sister without goiter (S.T.) but with almost the identical clinical syndrome; and has probably been shown in a distant cousin (S.R.), a feeble-minded goitrous cretin. This suggests that the basic disorder is a genetic defect in which the defective gene manifests irregular dominance or, less likely, is recessive in character. In the latter instance, both parents of the index patient, and both parents of the distant cousin would have to be carriers.

The circulating iodoproteins in these patients probably represent abnormal forms of thyroglobulin. There is more or less correspondence between

the I^{131} values for the thyroid gland hydrolysates and for the circulating iodoprotein fraction in Patient J.T., and good correspondence between the stable iodine values for the hydrolysates from the gland of J.T. and the hydrolysate of the iodoproteins of S.T. The circulating iodoproteins induced no autoantibodies against thyroglobulin, and upon enzymatic digestion they produced more thyroxine (T_4) than normal as well as a disturbed DIT:MIT ratio (12, 13). The shift in the DIT:MIT ratio in Patient J.T. is opposite to that found in carcinomatous glands (13). The findings in Patients J.T. and S.T. are in agreement with our previous results (7).

The fact that there was a similar distribution of iodoamino acids in the serum and in the thyroidal proteins suggests that the abnormal iodoproteins are synthesized in the gland and are thence discharged into the serum —a conclusion reached by DeGroot and coworkers (9) from their study of a similar patient. There is no indication from either study as to the physiologic process by which iodoproteins are released from the gland. Although the pathologic picture of the thyroid in Patient J.T. was somewhat different from that usually found in congenital goiter (14), there was no evidence of thyroiditis or of cellular disintegration. It is clear in the case of Patient S.T. that a goiter need not be present for such release to occur.

The results of replacement therapy in Patients J.T. and S.T. since the

TABLE 3. DISTRIBUTION OF IODOCOMPOUNDS IN THE THYROID GLAND OF PATIENT J. T. AND IN COMMERCIAL THYROGLOBULIN

Material	Approximate percentage distribution of I^{131} compounds				
	MIT	DIT	I^-	T_4	T_3
Right lobe of thyroid*	19	58	19	3	1
Left lobe of thyroid*	17	61	17	3	2
Human thyroglobulin†	44	49	6	1	>1
	Approximate percentage distribution of I^{127} compounds				
Right lobe of thyroid*	24	55	7	5	8
Left lobe of thyroid*	20	65	6	4	6
Commercial (beef) thyroglobulin*	33	48	4	10	5

* Digested with pancreatin followed by erepsin (12), 96 hours after labeling.

† Digested with trypsin (13). There is some evidence that trypsin does not liberate as much T_4 and T_3 as do pancreatin and erepsin.

laboratory investigation (see Case Reports) were those to be expected in uncomplicated hypothyroidism. This also would suggest that the defect originated within the thyroid and not from abnormal binding of thyronines in the periphery.

In addition to the iodoprotein abnormality, the data suggest a coexistent defect in the earlier stages of the hormonal synthetic cycle. The radioactivity in the butanol-soluble fraction of the serum of the goitrous cousin (S.R.) and of the original patient (J.T.) was present largely as iodide, with a corresponding decrease in the iodothyronine fraction. This could well be the consequence in Patient S.R. of the loss of functioning tissue. Disruption of hormonal synthesis in this subject was also indicated by the distribution of the serum I^{127} compounds; the values (MIT, 31 per cent; DIT, 43 per cent; I^-, 15 per cent; T_4, 8 per cent; T_3, 3 per cent) appeared to vary significantly from the average values for 8 apparently euthyroid individuals (MIT, 25 per cent; DIT, 27 per cent; I^-, 4 per cent; T_4, 35 per cent; T_3, 10 per cent (15)). An abnormal ratio between the labeled serum iodotyrosines and iodothyronines was also present in the presumably normal brother, H.T.

The story of possible exposure of the mother to radiation before marriage is probably not meaningful in terms of the thyroidal disorder in the children. No break in technic was known to have occurred at that time or according to regular checks for undue radiation exposure. Moreover, the development in 2 or possibly all 3 children of only a thyroid disorder without derangements elsewhere would be a most unusual consequence of radiation damage to the genes.

CASE REPORTS

Patient J. T. (UH #1365777)

This 6½-year-old boy was first seen November 9, 1957, because of "failure to grow properly." This had been recognized by 1½ years of age. The family history revealed that a distant cousin "took thyroid" when young. The mother had worked during World War II in a laboratory where there was possible risk of exposure to radiation. However, no accident was known and there was no evidence of undue exposure in regular surveys. Interspaced between children were numerous spontaneous abortions. There was no history of thyroid disease in the remainder of the family, although 7 maternal uncles and their 50 children were known to the patient's mother. The maternal grandfather had a brother and sister who were deaf-mutes, as well as a brother and sister who were healthy. The present patient's brother and sister had been termed normal until investigation revealed an organic defect in the patient. The sister was then stated to have the identical delay in growth and development that characterized the patient and was brought in for study. The family insisted, however, that the patient's brother was "normal in every way." The personal history and the record of previous health and illnesses were irrelevant except for the fact that the patient had had spinal meningitis at age 2, with no sequelae.

The presenting illness began at 18 months of age, when it was noticed that the infant was not growing as well as he should. He was considered normal at birth, and walked at

17 months. He did not speak until 2–3 years of age but spoke "whole paragraphs" then Currently he was considered unusually bright and the parents were urged by the school authorities to allow him to skip a grade.

Because of his increasingly evident shortness of stature from the age $1\frac{1}{2}$ years, the diagnosis of hypothyroidism had been entertained. Thyroid was given intermittently (0.015 Gm. to 0.09 Gm. daily) but none had been given for the preceding year and a half. In the eight months before he stopped this medication, he had grown 2 inches—a normal rate for his age. His first teeth had not been lost, his skin was rough, and he was constipated. No goiter had been observed at any time.

On physical examination the boy weighed 48 pounds (within 1 standard deviation (S.D.) of normal) and was 43 inches tall (-3 S.D.). Blood pressure was 96/74 mm. Hg. He appeared active, alert and bright. His face had a yellowish cast, and the skin generally was dry. The thyroid was definitely and diffusely enlarged to palpation; it was spongy and measured about $4.5\times2\times1$ cm. in each lobe. The testes were somewhat large, and each measured about $2\times1.5\times1$ cm. although the secondary sex characteristics showed no development. The liver was 3 cm. below the costal margin.

Laboratory findings: The hemoglobin level was 10.6 Gm. per 100 ml., and red blood cell count 3.4 million; the white blood cell and differential counts were normal. The 24-hour thyroidal I^{131} uptake was 67 per cent, and the serum protein-bound iodine level (PBI) was 3.4 μg. per 100 ml. Roentgenograms of the hands and wrists revealed an epiphyseal development consistent with a chronologic age of 3 years—a delay so marked that it was felt by the X-ray Department to be pathognomonic of hypothyroidism.

Biopsy and subtotal thyroidectomy were consented to after a series of consultations elsewhere. At operation performed April 7, 1958 by Dr. Lawrence W. Sloan of the Department of Surgery, "except for the fact that the gland was enlarged, it appeared to be quite normal. Perhaps there was some increase in fibrous tissue, for both lobes were rather firm. There was a pyramidal lobe from the right isthmus extending to the thyroid. Each lobe was approximately $4\times3\frac{1}{2}\times3$ cm. There were no suspicious nodes and no areas that felt like adenomas within the gland." The entire right lobe and part of the left lobe were removed for chemical and pathologic analysis.

Pathologic Report

The report which follows refers to the examination of the right lobe. The findings in the left lobe were virtually identical:

Gross: The specimen consists of a normally shaped but enlarged right lobe of the thyroid gland. It weighs 7 grams and measures $4\times3\times1.5$ cm. The surface is smooth, the consistency is normal and elastic. The surface of the gland and the cut surfaces have a red-brown, somewhat rusty color. The cut surfaces show a homogeneous finely lobular pattern with minimal focal increase in fibrous tissue.

Microscopic: The capsule is normal. There is a moderate increase of the interstitial tissue between lobules. The appearance varies somewhat from area to area. A few lobules show small or middle-sized follicles, usually without colloid or with dense laminated colloid. Most of the parenchyma is made up of solid nests of cells representing involuted follicles. The cells are cuboidal and large, with a finely granular cytoplasm of the Hürthle type. The nuclei are frequently bizarre, large and hyperchromatic. Fine golden-yellow or brownish, refractile pigment is seen in many solid nests or small follicles, scattered throughout the cytoplasm of the cells. This pigment is probably lipochrome. A few nodules of lymphoid tissue are scattered in the interstitium. No adenomatous structures are seen and there is no evidence of malignant changes.

The foregoing findings are evidence of involutive changes and inactivity. The bizarre anaplastic cells resemble those seen in adenomata developing in the thyroid of sporadic cretins.

Diagnosis: Diffuse goiter with involutive changes (hypothyroidism, clinically).

(Dr. W. A. Blanc)

Note: Radioautographs prepared on April 9, 1958 and exposed for eight days show sections labelled A & B, presumably representative sections of the right and left lobes of the gland. They show no uptake in the larger empty follicles described by Dr. Blanc, nor in the areas where the bizarre cells are seen and where the cells seem to be arranged in sheets. Uptake is spotty, but intense, in what, although they are somewhat obscured by the darkening on the photographic plate, seem to be very small follicles.

(Dr. V. Kneeland Frantz)

Progress Note

After operation, the patient grew $2\frac{1}{2}$ inches and gained $2\frac{1}{2}$ pounds. The bone age matured 1–2 years during thyroid therapy, 0.045 Gm. daily. Recently, the child seemed to feel better and to make better progress when taking triiodothyronine, 0.0375 mg. daily.

Patient S. T.

The patient was a girl aged 3 years 4 months, who weighed 6 pounds 1 ounce at birth, was cyanotic transiently, and had a "pot belly." She sat at the age of 8 months, walked at 18 months, but still said only "mama." The teeth were erupting "late," growth was slow, and her appetite was unusually poor. On physical examination she was $31\frac{3}{4}$ inches tall and weighed $30\frac{3}{4}$ pounds, appearing to be about $1\frac{1}{2}$ years old. The skin had a yellowish cast but was soft and smooth, and the pulse was 88. The liver was 5 cm. below the costal margin.

Laboratory findings: The hemoglobin level was 8.4 Gm. per 100 ml., red blood cell count 3.5 million, and white blood cell count 5,400 with the following differential count (per cent), PMN 24, L 68, M 4 and E 1. The serum PBI level was 4.0 μg. per 100 ml. Roentgenograms of the wrists revealed an epiphyseal age of 3–6 months "consistent with hypothyroidism." Determination of thyroidal I^{131} uptake was not permitted although blood was obtained for chromatography after a labeling dose.

With thyroid therapy, 0.045 Gm. daily from January 18 to December 29, 1958, the patient grew $3\frac{1}{4}$ inches but gained no weight. Her progress otherwise was described as "spectacular" by the family.

Patient S. R.

This 56-year-old male was the offspring of a consanguineous marriage between two cousins. He was recognized as a goitrous cretin at birth. He had spent most of his adult life in Letchworth Village because of feeble-mindedness. When seen, he was obviously mentally deficient, and was dressed as a child in a boy's knickerbockers and shirt. He was approximately 4 feet 8 inches tall and had a very large goiter. This was composed of a mass of nodules ranging in size from small to large. A number of tremendously large, deep and superficial dilated veins were visible in the thyroid region. Although I^{131} was administered for blood studies, no determination of thyroidal uptake was possible. The serum PBI level was 10.9 μg. per 100 ml. There was no evidence to suggest hyperthyroidism upon physical examination, despite the elevated PBI value.

Addendum

Since this article was submitted for publication, the studies by DeGroot and Stanbury (using ultracentrifugal and electrophoretic methods) of the butanol-insoluble material in this type of patient have appeared (16).

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THE EFFECTS OF PROGRESSIVE REDUCTION OF SODIUM INTAKE ON ADRENAL STEROID EXCRETION AND ELECTROLYTE BALANCE IN A CASE OF CONGENITAL ADRENAL HYPERPLASIA OF THE SALT-LOSING TYPE*

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ABSTRACT

Two balance studies, two and a half years apart, were conducted on a child exhibiting congenital adrenal hyperplasia of the salt-losing type. These studies were performed at the ages of 18 months and 4 years. The increased aldosterone excretion which occurred in response to restricted sodium intake and administration of ACTH was not associated with a positive sodium balance. Coincident with a negative sodium balance and elevated excretion of aldosterone the urine contained a biologically active fraction, obtained by paper chromatographic procedures, which promoted natriuresis and inhibited the sodium-retaining effect of desoxycorticosterone in the adrenalectomized rat.

THE syndrome of congenital adrenal hyperplasia has been well described with regard to its clinical and pathologic manifestations (1–5), biochemical characteristics (6, 7), genetic patterns (8) and the effects of treatment (1, 3, 9–14).

Elucidation of the cause of the electrolyte disturbance occurring in a subgroup of these patients presenting virilization and addisonian-like crises has not yet occurred.

In an attempt to shed some light on this particular metabolic defect, 2 balance studies, two and a half years apart, were performed in a child with

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congenital adrenal hyperplasia of the salt-losing type. These studies included observations on electrolyte balance and adrenal steroid excretion before, during and after provoked episodes of clinical crisis.

METHODS

Conventional balance techniques were employed. The composition of the diet was calculated from tables prepared by the Bureau of Human Nutrition and Home Economics, United States Department of Agriculture. Timed urine collections were made and checked for completeness by means of creatinine estimations.

Serum and urinary sodium and potassium were determined by flame photometry, using a Perkin Elmer model 52C instrument and an internal standard of lithium.

Porter-Silber chromogens were measured by the method of Silber and Porter (15).

For 17-ketosteroid analysis, urines were subjected to either strong hydrochloric acid (16) or enzymatic hydrolysis (17). Total urinary 17-ketosteroids were determined according to the method of Pincus (16).

Aldosterone determinations on urine were performed by hydrolysis at pH 1 with hydrochloric acid and at pH 5.2 enzymatically (18), followed by a chloroform extraction. The extracts were subjected to paper chromatography in the toluene-propylene glycol solvent system of Burton *et al.* (19). The cortisone zones were eluted and rechromatographed in Bush system C (20) to separate the aldosterone and cortisone zones. The aldosterone zones were eluted and the final quantitative estimations were carried out by a bioassay procedure (21, 22) and by a chemical method (23).

CLINICAL HISTORY

The patient described in these studies was, at the time of writing in April 1959, a $4\frac{1}{2}$-year-old male child with congenital adrenal hyperplasia of the salt-losing type. This diagnosis had been established at the age of 21 days on the basis of the clinical findings of diarrhea, lethargy, dehydration and cachexia, and the laboratory findings of hyponatremia, hyperkaliemia and elevated 17-ketosteroid excretion. He had been subsequently followed in the Endocrine Clinic of the Worcester City Hospital and admitted to the hospital on several occasions for additional studies. His clinical course and the details of these investigations will be published elsewhere. At the age of 18 months he was admitted for studies to clarify the metabolic defect with particular reference to electrolyte balance and steroid excretion patterns. He was readmitted at age 4 to the Metabolic Unit of the Worcester City Hospital for re-evaluation of the course of his disease and confirmation of previously obtained data.

STUDY I

Method of procedure. At 18 months of age an attempt was made to assess the effect of progressive restriction of sodium intake in the absence of steroid medication. The last dose of 25 mg. of desoxycorticosterone trimethylacetate had been administered two weeks before admission. Oral administration of hydrocortisone (15 mg. per day) was discontinued two days prior to the observation period. Balance data were accumulated over a nine-day period. During the first five days, sodium in the amount of 110 mEq. per day was added to a sodium-free, high-potassium diet. For the next three days, the amount of added sodium was 50 mEq. per day. On the

ninth experimental day, observations were made over a period of four and a half hours during which no sodium was administered.

Results. Table 1 shows the influence of varying amounts of salt intake on electrolyte balance. During the first five days of observation, with no steroid medication and with a sodium intake of 110 mEq. per day, a total loss of 434.0 mEq. of sodium occurred, although no clinical manifestations

TABLE 1. INFLUENCE OF SALT INTAKE ON ELECTROLYTE BALANCE

Day of study	Steroid medication	Intake per 24 hrs.		Urinary output per 24 hrs.			Sodium balance	Serum level (mEq./L)		Weight (Kg.)
		Fluids (ml.)	Na (mEq.)	Urine vol. (ml.)	Na (mEq.)	K (mEq.)		Na	K	
1	None	1,165	110	820	271.20	32.5	−161.20	141.2	4.6	12.47
2	None	1,305	110	1,032	290.16	67.0	−180.16			12.60
3	None	1,260	110	890	163.92	58.0	− 53.92			12.30
4	None	1,150	110	750	135.6	35.0	− 25.6			12.43
5	None	1,295	110	665	123.12	59.0	− 13.12	139.1	6.4	12.43
6	None	1,140	50	550	158.64	46.0	−108.64			11.92
7	None	880	50	340	64.40	48.0	− 14.40			11.99
8	None	920	50	400	69.84	26.0	− 19.84	132.6	6.6	11.77
9*	None	210	0	80	64.80	48.0	− 64.80	123.8	7.8	11.79

* Only a 4½-hour collection.

of electrolyte imbalance were noticeable. On the fifth metabolic day, there was a slight lowering of serum sodium concentration in association with an elevation of serum potassium concentration. On the sixth day the sodium chloride intake was decreased to 50 mEq. This intake was maintained for three days. Symptoms of anorexia, irritability and vomiting were observed on days 7 and 8. The serum sodium level was decreased on day 8 in association with a slight elevation of the serum potassium level. When no sodium was being administered (day 9), observation could be pursued for only four and a half hours due to marked irritability, vomiting, hypotension and an impending shock-like state. The serum sodium level dropped to 123.8 mEq. per liter, and the potassium level rose to 7.8 mEq. per liter. During the last four days of observation, a total loss of 207 mEq. of sodium and a 0.6-Kg. decrease in weight occurred.

Table 2 shows the pattern of steroid excretion. Control values for 17-ketosteroid (17-KS) excretion were obtained just prior to the experimental period. There was a fivefold increase in the excretion of 17-KS during the period of salt deprivation. Aldosterone was not detected by our method in samples corresponding to days 1–2, 6 and 8 of the study. Values of aldosterone excretion were within normal range in samples corresponding to days 5 and 7 but showed a marked elevation in the sample corresponding to day 9 when sodium was completely withdrawn. 17-Hydroxycorticosteroid (17-OH-CS) excretion patterns were not followed throughout the study. Two

weeks prior to admission, when the patient was receiving steroid medication (desoxycorticosterone trimethyl acetate and 30 mg. of hydrocortisone daily), the excretion of 17-OH-CS was 8.2 mg. per twenty-four hours. On day 9, however, when sodium was completely withdrawn from the diet, 17-OH-CS excretion fell below the levels obtained in normal children in our laboratory (1.20–1.92 mg. per twenty-four hours, up to 3½ years of

TABLE 2. STEROID EXCRETION PATTERNS

Day of study	Steroid medication	Dietary sodium (mEq.)	Urinary excretion		
			Aldosterone* (μg./24 hrs.)	17-KS (ketonic fraction) (mg./24 hrs.)	17-OH-CS (mg./24 hrs.)
Control	Desoxycorticosterone trimethylacetate, and 30 mg. oral hydrocortisone daily	200	—	0.29	—
		200	—	0.42	—
		200	—	0.21	—
		200	—	0.58	8.2
Day 1–2	None	110	<1	0.83	—
5	None	110	1.8	1.20	—
6	None	50	<1	—	—
7	None	50	3	—	—
8	None	50	<1	—	—
9†	None	None	18	1.99	0.36

* Final quantitative aldosterone determinations were carried out by means of a bioassay procedure. In our laboratory such values in normal children range from 0 to 3.5 μg./24 hrs.

† Only a 4½-hour collection as compared to 24-hour collections for the other assays.

age). Samples corresponding to days 1–2, 5, 6, 7, 8 and 9 were subjected to further chromatographic separation. Methods of procedure and results are described under "Special Studies."

STUDY II

Method of procedure. At the age of 4 years and 1 month a re-evaluation was made of the patient's response to sodium restriction in the absence of steroid medication. He had received 25 mg. of desoxycorticosterone trimethylacetate intramuscularly twenty-two days prior to admission and had been maintained with 15 mg. of oral hydrocortisone per day until four days prior to admission. During the period of observation, which lasted twenty-three days, the child was allowed normal activity about the ward. He was in bed approximately fourteen hours per day. His daily intake was

approximately 1200 calories with 1500 ml. of fluid. The sodium intake was between 134 and 163 mEq. per day during the first four days. For the next six days the sodium intake ranged from 77 to 99 mEq. per day. A nursing error occurred on the sixth day of the study, resulting in an increased intake. From the eleventh to the twenty-third day, a sodium intake of 134 to 163 mEq. per day was reinstituted coincident with exogenous steroid administration. An attempt was made to maintain an adequate but not excessive potassium intake. The patient's age and the fluctuations in his physical state made rigid control impossible.

Results. Table 3 shows the balance data obtained during the period of study. On the first metabolic day the child manifested some lethargy, anorexia and slight fever. The serum sodium level was 130 mEq. per liter. For that reason 20 mg. of hydrocortisone (compound F) and 3 mg. of desoxycorticosterone acetate (DCA) were administered; the symptoms disappeared. The following day the serum sodium level had returned to normal, the hematocrit was 45 per cent, hemoglobin level 12.3 Gm. per 100 ml., CO_2 combining power 26 mEq. per liter, serum nonprotein nitrogen (NPN) concentration 38 mg. per 100 ml., chlorides 90 mEq. per liter and fasting blood sugar level 110 mg. per 100 ml. Thereafter, a gradual reduction of dietary sodium was accomplished. During the first nine days of the study a progressive weight loss of 1.0 Kg. occurred. On the morning of the tenth day, in association with pronounced clinical signs including lethargy, hypotension and vomiting, the concentration of serum sodium dropped to 125 mEq. per liter and that of potassium rose to 6.0 mEq. per liter; the hematocrit rose to 47 per cent, the hemoglobin level to 13.6 Gm. per 100 ml., and the NPN level to 42 mg. per 100 ml. Although the overall negative sodium balance was only 175 mEq. during this period, symptoms of lethargy, anorexia and fussiness increased progressively. A negative potassium balance was also observed. This was accompanied by a progressive rise in the concentration of serum potassium at a time of restricted intake due to the clinical condition of the patient. In a similar patient deprived of steroid medication and receiving a low-sodium intake, these changes were attributed by Barnett and McNamara (24) to shifts of potassium from the intracellular to the extracellular compartments. A transfer of sodium in the opposite direction was probably occurring simultaneously.

Treatment with intravenous saline and 5 mg. of DCA was instituted at 2 p.m. on day 10. Clinical remission occurred almost immediately. Administration of DCA was continued for the remainder of the study in a dosage of 3 mg. per day. Positive sodium balance was maintained thereafter except on day 21. Throughout most of the study a negative potassium balance and slightly elevated serum levels persisted. There was a weight gain of 1.6 Kg.; values for hemoglobin, hematocrit, CO_2 combining power,

TABLE 3. SODIUM AND POTASSIUM BALANCE DATA

Day of study	Medication				Wt. (Kg.)	Intake (24 hrs.)		Urinary output (24 hrs.)			Balance		Serum level	
	Cpd. F* (mg.)	DCA (mg.)	ACTH (I.U.)	Progesterone (mg.)		Na (mEq.)	K (mEq.)	Urine vol. (ml.)	Na (mEq.)	K (mEq.)	Na (mEq.)	K (mEq.)	Na (mEq./L)	K (mEq./L)
1	20	3			18.8	137	16	679	192	115	−55	−99	130.0	—
2					19	163	55	861	146	55	+17	—	142.1	4.0
3					19	158	62	938	220	99	−62	−37	—	—
4					18.7	135	56	989	176	81	−41	−25	142.0	—
5					18.5	99	43	1,516	171	108	−72	−65	143.0	—
6					18.4	154	54	1,224	105	88	+49	−34	—	—
7					18.2	87	10.0	654	101	63	−14	−53	142.0	5.2
8					18.1	77	16	358	67	37	+10	−21	—	—
9					18.0	82	10.0	540	89	60	− 7	−50	140.0	6.0
10		5			18.3	89	1.0	503	60	35	+29	−34	125.0	6.0
11		5			18.3	106	30	1,159	59	51	+47	−21	135.0	5.1
12		3			18.7	107	41	1,622	59	70	+48	−29	135.0	4.5
13		3			19.0	166	80	1,172	79	49	+87	+31	—	—
14		3			19.2	145	60	903	90	51	+55	+ 9	138.0	—
15		3			19.2	131	52	834	127	57	+ 4	− 5	—	—
16		3	40		19.4	145	51	711	115	60	+30	− 9	136.0	5.4
17		3			19.6	120	47	909	101	66	+19	−19	130.0	5.8
18		3	40		19.5	107	50	1,101	97	68	+10	−18	142.0	5.5
19		3	40		19.7	115	56	1,369	117	82	− 2	−26	140.0	5.6
20		3			19.6	117	51	1,019	110	73	+ 7	−22	134.0	5.5
21		3		10	19.5	98	38	895	132	66	−34	−28	136.0	5.0
22	75	5			19.6	102	37	980	81	83	+21	−46	136.0	5.4
23	75	25†			19.6	113	44	1,084	121	79	− 8	−35	141.0	5.1

* Hydrocortisone.

† Desoxycorticosterone trimethylacetate.

and levels of NPN, chlorides and blood sugar remained within normal limits.

On the twenty-first day, a dose of 10 mg. of progesterone was given intramuscularly. This steroid, in doses of 50–150 mg., has been demonstrated to cause sodium excretion in normal adult subjects and in adult subjects with Addison's disease receiving aldosterone and cortisone (25). A change to negative sodium balance occurred on the day of administration. Because this was but a single observation, it cannot be considered significant.

On each of the sixteenth, eighteenth and nineteenth days, a dose of 40 units of ACTH was administered to determine the effect on steroid excretion. On the twenty-second and on the twenty-third day, a dose of 75 mg. of hydrocortisone was given; on the latter day, a dose of 25 mg. of desoxycorticosterone trimethylacetate was also given to prepare the child for discharge.

Table 4 shows the steroid excretion patterns. 17-KS excretion, in the absence of steroid administration, rose to about 16 mg. per day. ACTH caused no further increase. Reduction in 17-KS excretion followed the administration of large doses of hydrocortisone (compound F) prior to dis-

TABLE 4. STEROID EXCRETION PATTERNS

Day of study	Medication			Dietary intake (mEq./24 hrs.)		Urine vol. (ml.)	Urinary excretion (24 hrs.)			
	Cpd. F (mg.)	DCA (mg.)	ACTH (I.U.)	Na	K		Creatinine (Gm.)	Aldosterone* (μg.)	17-KS (ketonic fract.) (mg.)	17-OH-CS (mg.)
1	20	3		137	16	679	0.41	5.6	5.04	7.7
2				163	55	861	0.46	13.4		0.0
3				158	62	938	0.48	11.3		1.1
4				135	56	989	0.51	0.0		0.8
5				99	43	1,516	0.50	8.0		0.0
6				154	54	1,224	0.42	13.3		1.2
7				87	10.0	654	0.36	6.7		0.0
8				77	16	358	0.39	12.3		0.7
9				82	10.0	540	0.34	28.3	14.9	0.0
10		5		89	1.0	503	0.48	22.9		1.3
11		5		106	30	1,159	0.50	0.0		2.3
12		3		107	41	1,622	0.62	0.0		0.0
13		3		166	80	1,172	0.46	0.0		2.9
14		3		145	60	903	0.38	0.0	16.3	2.3
15		3		131	52	834	0.41	1.7		2.1
16		3	40	145	51	711	0.36	0.0	8.4	0.7
17		3		120	47	909	0.51	1.4		1.2
18		3	40	107	50	1,101	0.60	3.4		2.1
19		3	40	115	56	1,369	0.58	13.9		1.1
20		3		117	51	1,019	0.46	0.0	12.0	0.6
21†		3		98	38	895	0.63	1.7		2.1
22	75	5		102	37	980	0.64	0.0		22.8
23	75	25‡		113	44	1,084	0.48	10.7	3.84	17.3

* Final quantitative aldosterone determinations were carried out according to the method of Nowaczynski *et al.*
† A 10-mg. dose of progesterone was administered intramuscularly on day 21.
‡ Desoxycorticosterone trimethylacetate.

charge. 17-OH-CS excretion remained low during the observation period except when hydrocortisone was administered. Aldosterone excretion was variable during the period of gradual reduction of dietary sodium intake in the absence of steroid medication and reached a maximum on the ninth day, coincident with the appearance of severe symptoms. When a dose of 40 units of ACTH was administered for two consecutive days (days 18 and 19), a significant increase of aldosterone excretion occurred. Return to previous values followed until the twenty-third day, when desoxycorticosterone trimethylacetate (25 mg.) was administered and excretion rose again.

Samples corresponding to days 2 through 9 were subjected to further chromatographic separation. The method of procedure and results are described under "Special Studies."

SPECIAL STUDIES

The apparent paradox of the coincident occurrence of natriuresis with elevated aldosterone excretion suggested the existence of a substance which prevented aldosterone from exerting its physiologic effects. Studies were oriented towards the demonstration in this patient's urine of such a substance. Paper chromatographic and other techniques were used as described in the following sections. The eluted areas from paper chromatograms were tested in adrenalectomized male rats for biologic activity according to the method of Marcus *et al.* (21)—hereafter referred to as Method A—and of Kagawa *et al.* (22)—hereafter referred to as Method B. The characteristics of the assay method used are presented in Table 5.

The primary interest was to observe sodium-excreting or desoxycorticosterone (DOC)-inhibiting effects. The experimental design was the following: for each assay, different doses of the standard DOC, and varying doses

TABLE 5. RELATIVE POTENCY OF *dl*-ALDOSTERONE-21,MONOACETATE (A) AND DESOXYCORTICOSTERONE (DOC)

Method	Vehicle	End-point	Dose levels of A (μg.)	Number of rats	Dose levels of DOC (μg.)	Number of rats	Combined slopes	Index of precision	Significance of differences of slopes t (P)	Relative potency of A and DOC (range at P=0.95)
A	20% ethanol	Urinary Na excretion	0.12;0.24;0.48	8	4.2;8.4;16.8	8	1.27	.15	.13 >.5	25.31 (17.92–35.76)
B	20% ethanol	Urinary Na excretion	0.12;0.24;0.48	21	4.2;8.4;16.8	21	−3.52	.18	.28 >.5	31.11 (24.10–40.16)

of the eluted zones were used. The latter were administered separately and/or simultaneously (at two different sites) with 6 μg. or 25 μg. of DOC. Each dose (standard or unknown in combination with DOC) was given to a group of at least 8 animals, using 20 per cent ethanol as the injection vehicle.

Sodium excretion was calculated as the percentage of sodium excreted above values observed in control animals. DOC inhibition was calculated as the percentage inhibition of the sodium retention induced by the dose of DOC simultaneously tested.

Samples corresponding to days 1–2, 5, 6, 7, 8 and 9 (Study I)—hereafter referred to as Material 1—and samples corresponding to days 2 through 9 (Study II)—hereafter referred to as Material 2—were subjected to the following procedures: As described under "Methods," hydrolysis was carried out at pH 1 with hydrochloric acid and at pH 5.2 enzymatically (18), followed by chloroform extraction. Each extract was subjected to paper chromatography in the toluene-propylene glycol (TPG) system of Burton *et al.* (19) for seventy-two hours, to separate cortisone and steroids more polar than cortisone. The cortisone zones were eluted and aldosterone separated from cortisone and quantitatively determined (see "Methods"). The remainder of the 72-hour chromatogram corresponding to the portion below cortisone and the overflow were combined and re-run in the same system (TPG) for six to eight hours. Compound B and 11β-hydroxy-Δ^4-androstenedione were used as standards. A 2-mm. strip was cut from one edge of each paper chromatogram and was used to locate zones of Zimmermann or blue-tetrazolium reacting material.

Material 1. Chromatograms obtained from the six- to eight-hour TPG system, corresponding to samples 1–2, 5, 6, 7, 8 and 9, showed a positive Zimmermann zone extending from behind the corresponding area of standard corticosterone (compound B) (Rf: 1.5) to the corresponding area of standard 11β-hydroxy-Δ^4-androstenedione (Rf: 2.8). From each chromatogram the Zimmermann zone was eluted and the eluates were then pooled. From this material it was possible to crystallize about 20 mg. of an impure material which by infrared adsorption spectroscopy was demonstrated to contain 17α-hydroxypregnanolone as the major component. The sample was dispersed in potassium bromide. Identifying absorption bands occurred near 3600 (hydroxyl groups), 1700 (side-chain carbonyl), 1143, 1120, 1100, 1070, 1047, 1032, 980, 955 and 877 cm.$^{-1}$. Certain alterations in the spectrum indicated the presence of other minor components. The intensity of a band near 1250 was depressed, whereas that of bands near 1190 and 1160 was elevated. A weak new band could be detected near 868 cm.$^{-1}$, and diffuse broad absorption instead of sharp bands occurred near 1132, 990 and 805 cm.$^{-1}$.

A 6-μg. amount of this crystalline material was tested biologically against 6 μg. and 25 μg. of DOC, and produced 50 to 99 per cent inhibition of the DOC effect. Results are presented in Table 6.

Further work was carried out in an attempt to isolate the active substance from its impurities. Counter-current distribution was applied to this sample in a 100-cycle system. The solvents used were: upper phase, cyclohexane-ethyl acetate (65:35 by volume); lower phase, 50 per cent aqueous ethanol. The ratio of upper phase to lower phase was 1:1 by volume. Tubes 0–2 contained small amounts of residue; in tubes 3 to 20, traces of residue were present; tubes 21–29 did not contain any residue; tubes 30–38 contained some crystalline material; and tubes 45–68 contained crystalline material. Maximum concentrations were observed in tubes 57–65.

TABLE 6. METHOD A

Material administered		Urine vol. (ml.) ± SE	Na (mg./100 Gm./hr.) ± SE	K (mg./100 Gm./hr.) ± SE	Ratio K/Na ± SE	Per cent inhibition of Na excretion	P*	P**
Impure 17α-OH-prenanolone (μg.)	DOC (μg.)							
0	0	5.9±0.20	2.09±0.08	1.07±0.05	0.52±0.02			
	6	4.3±0.12	1.37±0.07	1.36±0.09	1.01±0.06		<.01	
	25	2.3±0.10	0.90±0.08	1.06±0.11	1.23±0.7		<.01	
6		5.4±0.5	2.14±0.11	1.20±0.19	0.55±0.07		>.5	
50		6.5±0.06	2.10±0.08	1.07±0.11	0.51±0.04		>.5	
6	6	5.6±0.80	1.82±0.08	1.55±0.33	0.84±0.15	63		<.01
6	25	4.4±0.28	1.50±0.12	1.54±0.15	1.08±0.15	50		<.01
50	6	6.5±0.50	2.08±0.16	1.42±0.15	0.69±0.04	99		<.01

* Significance of differences of mean values against controls.
** Significance of differences of mean values against DOC.

Tubes were pooled in series of 3 at a time, and the residues were tested for biologic activity. Each animal received 1/200 of the total sample. Tubes 21 through 35 showed a DOC-inhibiting effect varying from 58 to 116 per cent. Results are presented in Table 7. The crystalline material contained in tubes 54–74 did not show any biologic activity. The latter were pooled and the material recrystallized. The material had an infrared absorption spectrum and melting point (217°–220°) similar to that of a synthetic sample of 17α-hydroxypregnanolone.

Tubes containing the active material (Nos. 21–35) were pooled and attempts at crystallization were unsuccessful. Sublimation resulted in loss of biologic activity. No further work could be carried out.

Material 2. Chromatograms obtained from the six- to eight-hour TPG system demonstrated a positive Zimmermann zone running parallel to the corresponding area of the standard compound B. The paper chromatograms were then divided into several sections. Those areas corresponding to the standards (compound B and 11β-hydroxy-Δ^4-androstenedione) as well as those above and below, were eluted and tested separately for

biologic activity. Results are presented in Table 8. Those zones corresponding to the standard compound B (Zone II) and those with greater polarity than compound B (Zone I) caused 15 to 36 per cent inhibition of the DOC effect, and produced sodium-excreting effects ranging from 134 per cent (Zone II) to 141 per cent (Zone I). After completion of the biologic testing, the amount of material remaining was insufficient to carry out further isolation work.

Less total activity appeared to be present in Material 2 when the patient was 4 years old than in Material 1 when the patient was 18 months of age.

TABLE 7. METHOD A

Material administered		Urine vol. (ml.) ±SE	Na (mg./100 Gm./hr.) ±SE	K (mg./100 Gm./hr.) ±SE	Ratio K/Na ±SE	Per cent inhibition of Na excretion	P*	P**
CCD tubes No. (aliquot from total sample)	DOC (μg.)							
0	0	6.3±0.4	1.73±0.14	0.87±0.09	0.50±0.02			
	6	2.0±0.5	0.59±0.13	0.79±0.17	1.35±0.15		<.01	
21–22–23 $\left(\frac{1}{200}\right)$	6	3.2±0.5	1.25±0.03	0.55±0.09	0.44±0.07	58		<.01
0	0	5.6±0.4	1.82±0.15	0.75±0.06	0.41±0.01			
	6	3.8±0.3	1.21±0.16	0.54±0.06	0.46±0.01		.02	
24–25–26 $\left(\frac{1}{200}\right)$	6	8.9±2.6	2.11±0.39	1.28±0.20	0.79±0.12	116	>.1<.5	>.05<.1
30–31–32 $\left(\frac{1}{200}\right)$	6	8.8±2.0	1.97±0.25	1.05±0.15	0.53±0.03	108	>.5	>.02<.05
33–34–35 $\left(\frac{1}{200}\right)$	6	8.2±1.6	1.86±0.24	1.29±0.15	0.70±0.04	100	>.5	>.05<.1

* Significance of differences of mean values against controls.
** Significance of differences of mean values against DOC.

This seems to be consistent with the clinical observations that spontaneous disappearance of the salt-losing tendency occurs in some patients exhibiting this syndrome.

DISCUSSION

Two observations were made during these studies which may be significant. The first is that increased aldosterone excretion occurred under the stimuli of sodium restriction with the associated reduction of extracellular volume and the withdrawal of exogenous steroid medication. From our data it is impossible to determine whether only one or both of these stimuli were necessary to produce these results. The very important consideration as to whether aldosterone secretion is actually elevated under these circumstances has not been clarified by this work. Facilities for such deter-

TABLE 8. METHOD B

Material administered		DOC (μg.)	Total bladder content (mg.)		Ratio K/Na ±SE	Per cent change in Na excretion	P*	Per cent inhibition of Na excretion	P**
Aliquot from total sample									
Zone I	(Zone II		Na ± SE	K ± SE					
0	0	0	4.16±1.8	3.5 ±2.8	0.85± 0.07				
		3	2.68±0.8	4.7 ±0.9	2.94± 1.4	−36	>.1 <.5		
		6	0.14±0.03	3.18±1.7	21.7 ±10.8	−97	.05		
1/500	—	6	0.75±0.2	5.30±0.9	11.75± 4.0			15	>.02<.05
—	1/500	6	1.64±0.6	7.33±1.1	7.02± 1.9			36	>.02<.05
0	0	0	3.28±0.4	3.35±0.3	1.09± 0.2				
		3	2.80±0.3	4.87±0.4	1.88± 0.3	−15	>.1 <.5		
		12	0.84±0.10	5.70±0.3	7.59± 1.7	−74	<.01		
1/500		—	4.62±0.5	4.56±0.3	1.05± 0.2	+41	>.05<.1		
	1/500	—	4.39±0.5	3.74±1.0	1.05± 0.2	+34	>.05<.1		

* Significance of difference of mean values against controls.
** Significance of differences of mean values against DOC.

minations according to the method of Ayres *et al.* (26) were not available at the time of these studies. Control data in children of this age under similar experimental conditions would help to clarify the state of aldosterone synthesis and metabolism in these patients. Unfortunately, we were unable to perform such studies concomitantly.

Adrenocorticotropin (ACTH) has been shown to play a role in aldosterone regulation in normal subjects (27). In the adrenogenital syndrome high blood levels of ACTH have been demonstrated (28). Patients with only the masculinizing aspects of this syndrome exhibit mildly elevated aldosterone excretion levels which become further elevated during sodium restriction (29). In those patients with an associated sodium-losing tendency, baseline values for aldosterone excretion have been variously reported as norma- (29) or increased (30). If normal excretion persists, the question of a biol synthetic block preventing the aldosterone response to endogenously high corticotropin levels, is raised.

In patients exhibiting the salt-losing type of the adrenogenital syndrome there is an excessive production of androgens, as reflected by a high excretion of urinary 17-KS. Associated with the excessive production of androgens, there is also a deficiency in the biosynthesis of cortisol. These findings have been documented in studies showing markedly diminished excretion of the urinary metabolites of cortisol, tetrahydrocortisol and tetrahydro-

cortisone (31). The ability of our patient's adrenals to produce 17α-hydroxycorticoids, with no increase under conditions of stress, is in accordance with the foregoing observations and seems to exclude, in this case, an absolute deficiency in the 21-hydroxylase system. This postulate is further supported by the demonstration that aldosterone excretion rose in response to restriction of sodium and to administration of ACTH. The possibility that variants of this syndrome may demonstrate disparate alterations in aldosterone metabolism warrants further study.

Secondly, the demonstration of the lack of an appropriate sodium retention coincident with the increase in aldosterone excretion suggested the existence of some mechanism preventing aldosterone from exerting its full physiologic effect. By paper chromatographic procedures, it was possible to separate from the urine of this patient a fraction which led to natriuresis and which prevented DOC from exerting its sodium-retaining effect in the adrenalectomized rat. Further studies of this fraction are being pursued. This observation confirms a similar one made by Neher *et al.* (32) and by Klein *et al.* (33), and appears to bear etiologic significance in this particular syndrome.

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MODIFICATION OF THE EFFECTS OF ALDOSTERONE ON ELECTROLYTE EXCRETION IN MAN BY SIMULTANEOUS ADMINISTRATION OF CORTICOSTERONE AND HYDROCORTISONE. RELEVANCE TO CONN'S SYNDROME

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ABSTRACT

In a normal subject, the simultaneous administration of corticosterone and aldosterone consistently resulted in a greater potassium diuresis than did the administration of aldosterone alone, both when the steroids were administered during the day when spontaneous variation of potassium excretion due to diurnal rhythm is great, and during the night when variation due to diurnal rhythm is minimal. It is suggested that this may help to explain the pronounced potassium wastage seen in Conn's syndrome with high secretion of corticosteroids other than aldosterone (*e.g.*, corticosterone) compared with cases of secondary hyperaldosteronism in which secretion of other corticosteroids is not excessive.

IN 1955, Conn (1) described a syndrome of hypertension associated with hypokaliemia which was attributed, without direct evidence, to the secretion of excessive quantities of aldosterone. Subsequently, many examples of this syndrome have been reported, in some of which an actual excess of aldosterone has been demonstrated in the urine, although positive chemical identification of the sodium-retaining factor as aldosterone has been made in very few instances.

Striking features of this syndrome of "primary aldosteronism" are the excessive renal wastage of potassium and the absence of edema. On the other hand, these features are not found in cases of "secondary aldosteronism," in which the amount of aldosterone secreted and excreted is often greater than occurs in "primary aldosteronism." The effect on electrolyte excretion resulting from the administration of aldosterone to normal subjects for periods of two to four weeks has been reported by August, Nelson and Thorn (2). In one subject, the combined loss of potassium in the urine and feces did not exceed the potassium intake; in the other subject, there was a mean negative potassium balance of 9.7 mEq. per day. Thus even in

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daily doses of 3 to 6 mg. of the racemic acetate, aldosterone did not consistently give rise to a potassium deficit. Nevertheless, potassium depletion is a consistent and striking feature of Conn's syndrome. An excessive production of other adrenocortical steroids, in particular of corticosterone, has been reported in cases of Conn's syndrome (3, 4), whereas in secondary aldosteronism the production of other adrenocortical hormones is normal. Since the excessive secretion of corticosterone may modify the effect of aldosterone on the renal handling of sodium and potassium, a comparison has been made of the effects of aldosterone alone, and of the simultaneous administration of aldosterone and corticosterone, on the excretion of sodium and potassium in a normal subject. Data concerning the modification of the effect of *dl*-aldosterone monoacetate on the renal excretion of sodium and potassium by the simultaneous infusion of hydrocortisone, previously reported (5), will also be discussed.

METHODS

Normal subject

(a) *Intravenous infusion of aldosterone and corticosterone during the daytime.* Urine was collected from a normal subject (the author) at hourly intervals from 7 A.M. until 10 P.M. and then at intervals until 7 A.M. the following morning. Two-hourly feedings were given from 8 A.M. to 10 P.M., each consisting of 3 cream crackers, butter, cheese and 200 ml. of an orange carbonated drink. Each meal contained 12 mEq. of sodium and 2 mEq. of potassium. The low potassium content of the feedings was an error, since it was not realized until the diet had been analyzed how low was the potassium content of the orange drink. On three occasions, separated by intervals of at least a week, an eight-hour intravenous infusion of 5 per cent glucose-water was given from 9 A.M. to 5 P.M. On one occasion, 1 mg. of *dl*-aldosterone monoacetate dissolved in 10 ml. of ethanol was added to the glucose; on another occasion 1 mg. of *dl*-aldosterone monoacetate plus 100 mg. of corticosterone (free alcohol) dissolved in 10 ml. of ethanol was added; and on the third occasion the addition consisted of 10 ml. of ethanol alone (control).

(b) *Intramuscular injection of aldosterone and corticosterone over 24-hour period.* Urine from the same subject was collected over 24-hour periods, from 7 A.M. to 7 A.M. daily. The food intake was as constant as could be managed on a home diet. Eight successive 24-hour specimens of urine were collected as controls, to assess day-to-day variations. Corticosterone (25 mg. in arachis oil) was then given intramuscularly at 7 A.M. and 7 P.M. At intervals of four days, a dose of 200 μg. of *dl*-aldosterone monoacetate in arachis oil was given twice daily, or corticosterone plus aldosterone in the dosage mentioned in paragraph (a). Sodium, potassium and creatinine concentrations were estimated on these 24-hour samples.

(c) *Intramuscular injection of aldosterone and corticosterone during the night.* The intravenous infusions described in paragraph (a) were given over a period during the day when changes in sodium and potassium excretion are maximal as the result of diurnal variation of electrolyte excretion. Since it is preferable to carry out this type of investigation when changes in electrolyte excretion due to diurnal variations are minimal, further studies were performed on the same subject during the night. Urine samples were collected at two-hourly intervals, to determine the pattern of the diurnal rhythm of sodium and potassium excretion. The minimum was between 11 P.M. and 7 A.M. Urine was there-

fore collected from 11 P.M. to 7 P.M. daily over a period of twenty days, to determine day-to-day fluctuation. The rate of excretion of sodium showed some variation with temperature and humidity, but the rate of excretion of potassium was remarkably constant. The food intake was as constant as could be managed on a home diet; the last meal was taken at 7:30 P.M. Intramuscular injections of *dl*-aldosterone monoacetate (200 μg. in arachis oil), of corticosterone (free alcohol, 25 mg. or 12.5 mg. in arachis oil), or of both together, were given at 11 P.M. at intervals of at least four days.

Sodium and potassium concentrations were measured by flame photometry. Creatinine was determined by the method of Bonsnes and Taussky (6).

Addison's disease

The patients with Addison's disease mentioned in the discussion received a constant diet containing 180 mEq. of sodium and 150 mEq. of potassium. No steroid therapy was given on the day of the infusion or for the two previous days.

Patient W.G. was a 3-year-old male with Addison's disease of seven years' duration. He was normally maintained with 12.5 mg. of cortisone and 0.2 mg. of fluorohydrocortisone daily. His basal excretion of 17-hydrocorticoids was 1.7 mg. per twenty-four hours, and there was no response to corticotropin.

Patient R.S. was a 58-year-old female with Addison's disease of fifteen years' duration. She was normally maintained with 37.5 mg. of cortisone daily and 25 mg. of deoxycorticosterone trimethylacetate monthly. Her basal excretion of 17-hydroxycorticoids was 1.6 mg. per twenty-four hours, and there was no response to corticotropin.

RESULTS

Effect of intravenous infusion of aldosterone and corticosterone

The effect of these infusions upon the renal excretion of sodium and potassium is shown in Figure 1. During the 24-hour period from the commencement of the infusion, the addition of aldosterone resulted in a reduction of sodium excretion to 42 per cent of the control (glucose+alcohol), and an increase in potassium excretion to 181 per cent of the control. The simultaneous infusion of corticosterone with aldosterone slightly reduced the effect of aldosterone on sodium excretion (85 per cent of the control), but greatly increased the effect of potassium excretion (190 per cent of the control). The data are listed in Table 1.

The effects of the two hormones, alone and together, on sodium and potassium excretion during the actual eight-hour period of the infusion are also shown in Table 1. With aldosterone alone, sodium excretion fell to 42 per cent of the control; with aldosterone plus corticosterone, it was 84 per cent of the control. With aldosterone alone, potassium excretion was 233 per cent of the control; with aldosterone plus corticosterone it increased to 281 per cent of control.

Previous studies (5) showed that aldosterone, infused intravenously, had very little effect on the excretion of sodium and potassium during the first two hours of infusion. If only the last six hours of the infusion period are considered, the change in sodium and potassium excretion is even more

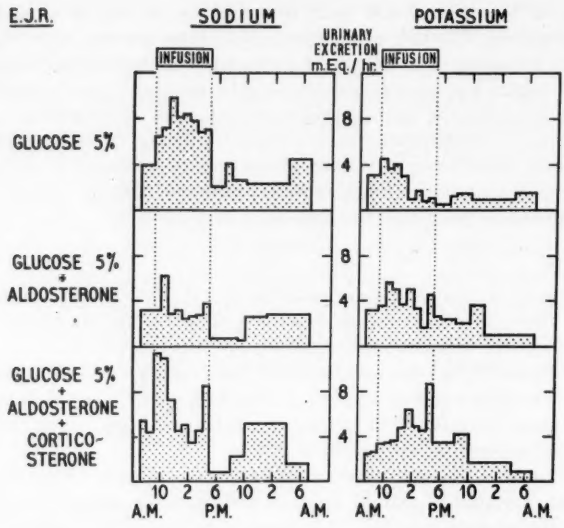

FIG. 1. Changes of sodium and potassium excretion in a normal subject during eight-hour infusions of glucose alone (control), glucose plus aldosterone, and glucose plus aldosterone plus corticosterone.

striking (Table 1). Sodium excretion fell to 39 per cent of the control when aldosterone was given, but was 70 per cent of the control when aldosterone plus corticosterone were given. Potassium excretion rose to 255 per cent of the control with aldosterone alone, and to 353 per cent of the control with aldosterone plus corticosterone.

Effect of intramuscular injection of aldosterone and corticosterone over 24-hour period

The modification of the urinary excretion of sodium and potassium that occurred over a 24-hour period during injection of aldosterone, and of aldosterone plus corticosterone, is shown in Table 2. The mean excretion on eight control days was 159.5 ± 10.1 mEq. of sodium and 78.0 ± 7.2 mEq. of potassium. The intramuscular administration of 200 μg. of *dl*-aldosterone monoacetate reduced sodium excretion to 74.9 mEq. (47 per cent of the control) and increased potassium excretion to 85.4 mEq. (109 per cent of the control). When aldosterone and corticosterone (50 mg.) were administered, the excretion of sodium was 107 mEq., and of potassium 97.5 mEq. The administration of corticosterone in addition to aldosterone thus modified the effect due to aldosterone alone, increasing the amount of sodium excreted, but also increasing the potassium loss.

TABLE 1. EFFECT OF INTRAVENOUS ADMINISTRATION OF ALDOSTERONE, AND OF ALDOSTERONE PLUS CORTICOSTERONE, ON EXCRETION OF SODIUM AND POTASSIUM IN A NORMAL SUBJECT DURING VARIOUS PERIODS OF TIME

Time period	Electrolyte (mEq./24 hrs.)	Control* period	Aldosterone* period	Aldosterone + corticosterone* period
24 hours	Sodium	132.0	55.3	112.6
	Potassium	40.2	72.8	77.0
8 hours	Sodium	67.3	28.6	56.2
	Potassium	14.5	33.7	41.4
6 hours	Sodium	48.6	18.9	33.8
	Potassium	9.5	24.2	34.5

* The control infusion was 400 ml. of 5% glucose-water+10 ml. of ethanol, given intravenously over a period of 8 hours. *dl*-Aldosterone monoacetate (1 mg. dissolved in 10 ml. of ethanol and 400 ml. of 5% glucose-water), or corticosterone (free alcohol, 100 mg.) plus *dl*-aldosterone monoacetate (1 mg.) dissolved in 10 ml. of ethanol and 400 ml. of 5% glucose-water, were infused on separate occasions over the same time-intervals.

Effect of intramuscular injection of aldosterone and corticosterone during the night

The mean excretion of sodium in the urine from 11 P.M. to 7 A.M. over a control period of twenty days was 7.84 mEq. per hour, with a standard deviation of 0.48. The mean potassium excretion during the control period was 2.36 mEq. per hour, with a standard deviation of 0.11. This resulted in a urinary sodium/potassium ratio of 3.32.

TABLE 2. EFFECT OF INTRAMUSCULAR ADMINISTRATION OF ALDOSTERONE, AND OF ALDOSTERONE PLUS CORTICOSTERONE, ON 24-HOUR EXCRETION OF SODIUM AND POTASSIUM IN A NORMAL SUBJECT

Electrolyte	Control* period	Aldosterone† period	Aldosterone +corticosterone‡ period
Sodium (mEq./24 hrs.)	159.5±10.1	74.9	107.0
Potassium (mEq./24 hrs.)	78.0± 7.2	85.4	97.5

* Mean and S.D. of eight 24-hour urine collections.

† 200 μg. of *dl*-aldosterone monoacetate by intramuscular injection.

‡ 200 μg. of *dl*-aldosterone monoacetate+50 mg. of corticosterone (free alcohol) by intramuscular injection.

The administration of corticosterone significantly reduced the excretion of sodium ($P = 0.01$) and increased the excretion of potassium ($P < 0.01$). The administration of aldosterone likewise significantly decreased the excretion of sodium ($P < 0.01$) and increased that of potassium ($P < 0.01$). The simultaneous administration of 200 μg. of aldosterone and 12.5 mg. of corticosterone further reduced the excretion of sodium compared with that of aldosterone, but had no additional effect on potassium excretion. On the other hand, the administration of 25 mg. of corticosterone with 200 μg. of aldosterone both reduced the sodium excretion (by 19 per cent) and considerably increased the potassium excretion (by 29 per cent), compared with the occasion when aldosterone alone was administered. These results are summarized in Table 3.

TABLE 3. MODIFICATION OF SODIUM AND POTASSIUM EXCRETION IN A NORMAL SUBJECT BY SIMULTANEOUS INTRAMUSCULAR INJECTION OF CORTICOSTERONE AND ALDOSTERONE DURING THE NIGHT

	Control*	Corticosterone (25 mg.)	Aldosterone (200 μg.)	Aldosterone (200 μg.) +corticosterone (12.5 mg.)	Aldosterone (200 μg.) +corticosterone (25 mg.)
Sodium (mEq./hr.)	7.84	6.4	5.2	4.8	4.2
Potassium (mEq./hr.)	2.36	3.1	3.8	3.8	4.9
Na/K ratio	3.30	2.1	1.4	1.2	0.85
Creatinine (mg./min.)	1.11 ± 0.0095	1.17	1.16	1.23	1.17

* Mean of 20 control urine collections from 11 P.M. to 7 A.M.

DISCUSSION

These studies show that the administration of corticosterone simultaneously with aldosterone considerably modifies the effects of aldosterone on the urinary excretion of sodium and potassium.

When the steroids were administered by the intravenous route, corticosterone given simultaneously with aldosterone not only decreased the sodium-retaining effect of aldosterone, but also enhanced very considerably the potassium-excreting properties of the hormone. This is well demonstrated in the data on the eight-hour infusions, and is even more striking if the first two hours of the infusion period are ignored—a stratagem which is justified by a previous study of the time relationships of steroid action (5). This effect is also seen, but to a lesser extent, over the 24-hour period following intramuscular injection of the two steroids.

A similar result was obtained when the steroids were administered over

a period of the day (11 P.M. to 7 A.M.) when alteration in excretion of sodium and potassium due to diurnal rhythm was minimal. Comparison of day-to-day excretion of electrolytes was more valid by this means than when the study was conducted during the more usual working hours. The aim was to administer amounts of corticosterone which were approximately five and ten times the normal secretion rate; this was estimated as 2 to 3 mg. a day (7). At the same time the amount of aldosterone administered was not greatly in excess of the normal secretion. It was thus hoped to simulate conditions existing in some cases of Conn's syndrome. A diurnal variation of excretion, and so presumably of secretion, of aldosterone has been reported (8) by Hernando *et al.*, the amount produced during the day being about twice that produced by night. The normal daily secretion rate of aldosterone has been variously estimated as 170 to 190 μg. (9), 230 to 350 μg. (10) or 82 to 190 μg. (11). The amount of *dl*-aldosterone monoacetate administered during our studies was equivalent to 92 μg. of the active hormone, approximately the amount secreted during the eight hours under study. In addition there was the subject's endogenous secretion. Under these conditions, the administration of 12.5 mg. of corticosterone did not augment the increase in excretion of potassium resulting from the injection of aldosterone alone, although it did result in a reduction of sodium excretion. The administration of 25 mg. of corticosterone, ten times the normal daily secretion, resulted in a considerable increase in the excretion of potassium, as well as a further reduction of sodium excretion.

With these results in mind, review was made of the modifications of sodium and potassium excretion in the urine of 2 patients with Addison's disease (previously reported (5)), caused by the simultaneous infusion of aldosterone and hydrocortisone. Table 4 shows the relevant data. The intravenous administration of hydrocortisone (free alcohol) at a rate of 20 mg. over eight hours consistently augmented the excretion of potassium, compared with the increase caused by the infusion of aldosterone alone. This is particularly well seen during the last six hours of the infusion, when the increase due to hydrocortisone was 43 per cent in Patient W.G. and 46 per cent in Patient R.S.

It will be seen from Table 4 that the effect of the addition of hydrocortisone on sodium excretion was not consistent in the 2 subjects. In Patient R.S., hydrocortisone reduced the sodium-retaining effect of aldosterone. In Patient W.G., it had this effect over the six-hour period, but not over the other periods.

These results may help to explain the excessive potassium wastage that occurs in Conn's syndrome—a phenomenon which has disturbed others(12) as well as ourselves (13). The explanation for this is not straightforward in terms of the known physiologic properties of aldosterone. It stresses the

TABLE 4. EFFECT OF INTRAVENOUS ADMINISTRATION OF ALDOSTERONE, AND OF ALDOSTERONE PLUS HYDROCORTISONE, ON EXCRETION OF SODIUM AND POTASSIUM IN PATIENTS WITH ADDISON'S DISEASE DURING VARIOUS PERIODS OF TIME

Time period	Patient	Electrolyte (mEq./24 hrs.)	Control* period	Aldosterone* period	Aldosterone +hydrocortisone* period
24 hours	W.G.	Sodium	184.6	155.9	107.8
		Potassium	50.4	95.9	139.0
	R.S.	Sodium	108.2	84.5	95.5
		Potassium	49.4	80.1	109.7
8 hours	W.G.	Sodium	91.2	39.2	31.6
		Potassium	18.2	41.6	56.4
	R.S.	Sodium	52.0	33.1	57.1
		Potassium	18.4	34.5	47.7
6 hours	W.G.	Sodium	70.2	16.2	20.4
		Potassium	14.0	37.3	53.5
	R.S.	Sodium	38.4	19.8	40.9
		Potassium	13.4	28.9	42.1

* The control infusion was 400 ml. of 5% glucose-water plus 10 ml. of ethanol, given intravenously over a period of 8 hours. *dl*-Aldosterone monoacetate (2 mg. dissolved in 10 ml. of ethanol and 400 ml. glucose-water), or hydrocortisone (free alcohol, 20 mg.) plus *dl*-aldosterone monoacetate (2 mg.) dissolved in ethanol and 400 ml. of glucose-water were infused over the same time-interval on separate occasions.

need to investigate abnormalities of secretion of adrenocortical steroids other than aldosterone in cases of Conn's syndrome, particularly when the rate of secretion of aldosterone is within the normal range.

The question of the mechanism involved in the observed modification in electrolyte excretion will not be discussed, except to note that apparently it is not due to an increased glomerular filtration rate, since this function (as crudely measured by creatinine clearance) was not significantly altered by the administration of corticosterone.

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THE METABOLISM OF 17α-HYDROXYPROGESTERONE AND ITS RELATION TO CONGENITAL ADRENAL HYPERPLASIA*

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ABSTRACT

17α-Hydroxyprogesterone was administered orally to 4 patients, including 1 adult with congenital adrenal hyperplasia. Chromatographic analysis of the urinary metabolites obtained after enzymatic hydrolysis yielded four pregnanetriol isomers, three 17-hydroxypregnanolone isomers, androsterone, epiandrosterone, etiocholanolone, androstanedione, etiocholanedione and two $C_{19}O_3$ compounds (11-hydroxyandrosterone and 11-ketoetiocholanolone). It is concluded that the metabolism of 17α-hydroxyprogesterone proceeds by way of full reduction of the Δ^4-3-keto group, followed by reduction of the C_{20}-ketone group and finally by cleavage of the side-chain. Considerable individual variation was observed with respect to the proportion of C_{21} and C_{19} compounds excreted and also with respect to the proportions of the individual isomers of the C_{21} compounds. The metabolites of 17α-hydroxyprogesterone do not appear to account for the masculinization seen in congenital adrenal hyperplasia; this and the disproportionately high excretion of androsterone in this disorder are highly suggestive of an associated overproduction of adrenal androgens, presumably dehydroisoandrosterone, Δ^4-androstenedione and 11-oxygenated Δ^4-androstenedione.

17α-Hydroxyprogesterone is known to be an intermediate compound formed during the biosynthesis of adrenal (1) and gonadal (2–4) hormones. Furthermore, it has been implicated as the possible androgen or source of androgens in congenital adrenal hyperplasia. Its metabolic fate in the body has therefore assumed considerable theoretical and clinical importance.

Early studies on the administration of this compound were limited to a measurement of quantitative changes in urinary 17-ketosteroid excretion (5, 6) or to the assessment of biologic activity, of which there appears to be relatively little in animals (7–11) and apparently none in man (12). The work of Bartter *et al.* (13) and of Wilkins and his group (14) on the abnor-

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malities of steroid biosynthesis in congenital adrenal hyperplasia gave added impetus to the study of these intermediate steroids. Qualitative investigation of the urinary metabolites of 17α-hydroxyprogesterone was undertaken by several workers (15–19) in normal subjects as well as in patients with a variety of endocrine diseases. In most instances, however, techniques were employed which are now known to result in artefact formation and partial destruction of important compounds such as pregnanetriol. Therefore it was decided to re-examine the urinary steroid pattern resulting from the oral administration of 17α-hydroxyprogesterone to selected patients using the mildest physicochemical techniques available, and to attempt a correlation of the findings with the urinary steroid pattern in congenital adrenal hyperplasia before and during the administration of this compound.

MATERIALS AND METHODS

Clinical material

Four patients were used in these studies.

V.S. (*Case 1*) was a woman physician with classic pituitary infantilism, secondary hypothyroidism and ovarian hypofunction. Control urinary 17-ketosteroid excretion ranged from 5.5 to 7.4 mg. per twenty-four hours, and corticoid (Porter-Silber chromogen) excretion ranged from 2.9 to 6.7 mg. per twenty-four hours. While the patient was taking an oral maintenance dosage of 0.2 mg. of *l*-thyroxine daily, a two-day pool of control urine was obtained; the 24-hour excretion of 17-ketosteroids was 5.3 mg., and of corticoids 2.9 mg. She was then given 100 mg. of 17α-hydroxyprogesterone daily by mouth for a period of twenty days, and urine was collected and pooled during the last four days of this treatment. The 24-hour excretion of 17-ketosteroids averaged 23.1 mg., and of corticoids 5.6 mg.

W.S. (*Case 2*) was a 12-year-old boy with mild hypopituitary hypogonadism, whose excretion of 17-ketosteroids averaged 11.7 mg. per twenty-four hours, and of corticoids 6.7 mg. per twenty-four hours. He received a single daily oral dose of 100 mg. of 17α-hydroxyprogesterone for one hundred and two consecutive days without visible clinical effect (12). 17-Ketosteroid excretion remained between 10.1 and 13.5 mg. per twenty-four hours except for a single rise to 33.6 mg. on the twenty-fifth day of therapy. Corticoid excretion ranged from 1.5 to 5.4 mg. per twenty-four hours. Urine collections were combined into a two-day and a five-day pool (including the urine collected on day 25); these were extracted and subjected to chromatography.

U.R. (*Case 3*) was a 26-year-old woman with Sheehan's syndrome who had had a single episode of vaginal staining about two months before admission. The 24-hour excretion of 17-ketosteroids averaged 3.6 mg., and of corticoids from 0.5 to 1.3 mg. While taking a daily dose of 10 μg. of triiodothyronine, she was given 100 mg. of 17α-hydroxyprogesterone twice daily for thirty-two days. After a control period of fourteen days, the same dosage was administered for twenty-eight days. The urinary excretion studies are shown in Figure 1. A two-day control urine, a six-day pool of urine collected during treatment, and a two-day pool from the interval control period were analyzed chromatographically. It was subsequently discovered that the patient was in the first trimester of pregnancy during these investigations.

G.S. (*Case 4*) was a 29-year-old female with congenital adrenal hyperplasia of the

normotensive type. Control 17-ketosteroid excretion ranged as high as 100 mg. per twenty-four hours, and corticoid excretion was about 2.8 mg. but could be elevated to 6.8 mg. per twenty-four hours with an injection of 50 units of gel-ACTH. Steroid suppression therapy was withheld for a period of eight weeks, and 17α-hydroxyprogesterone was then administered orally in a dosage of 100 mg. three times daily for six days, followed by 100 mg. twice daily for eight days. A control 24-hour urine obtained just before, and a pool of 2 urines collected during administration of 17α-hydroxyprogesterone were analyzed.

Methodology

Urines were collected without preservative and stored at 4° C. until preliminary quantitative studies were completed. 17-Ketosteroid determinations were performed with the aqueous alcohol technique, using amyl acetate extraction of the chromogen (20) to

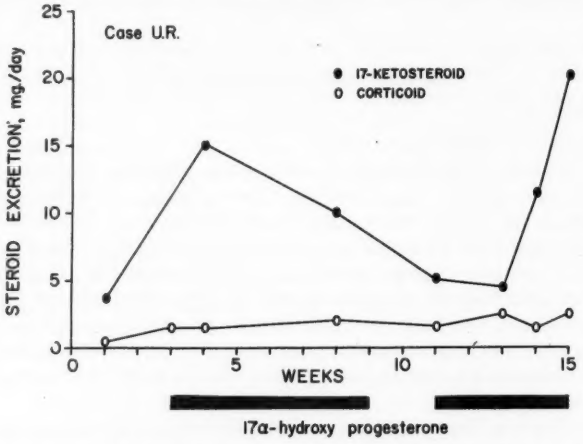

FIG. 1. Excretion of 17-ketosteroids and Porter-Silber chromogens (corticoids) during the administration of 17α-hydroxyprogesterone in Case 3 (U.R.).

remove spurious colors. Corticoids were measured by the Porter-Silber reaction applied to chloroform extracts of urine treated by glucuronidase hydrolysis. For total steroid extractions, urine was adjusted to pH 4.5 with acetic acid and acetate buffer; following addition of 200 units of glucuronidase (Ketodase) per ml. of urine, the material was incubated at 37° C. for a minimum of forty-eight hours. The product was subjected to extraction with chloroform or ethyl acetate, and the extracts were evaporated in a rotating flash evaporator at 45° C. under nitrogen, yielding tarry residues. The phenolic and acidic materials were removed from either solution of the residues by extraction with saturated sodium bicarbonate and *N* sodium hydroxide. The spent urine was boiled for fifteen minutes with 15 volumes per cent of concentrated hydrochloric acid and extracted with chloroform; these extracts were processed separately. The glucuronidase and the acid-hydrolysis extracts were divided into suitable aliquots and chromatographed on Whatman No. 1 filter paper according to the methods of Zaffaroni and Burton (21) and Axelrod (22–24). The steroid-containing zones were visualized by means of a modified Zimmermann test (22), 2,4-dinitrophenylhydrazine (22), blue tetrazolium (21) and fuming sulfuric acid (15 per cent free SO_3) (24). Various micro-organic reactions

were performed on the paper strips to determine the types of side-chain present (25). Each purified zone resulting from the chromatographic analysis was eluted and dried *in vacuo* under nitrogen at 45° C. The fractions were further purified by removing traces of pigment and background materials derived from the filter paper. At this point attempts were made to obtain at least one crystalline crop from each sample, and a melting point, using a Nalge-Axelrod micro melting-point apparatus.

A portion of the crystalline or semicrystalline material was dissolved in absolute methanol and the absorption spectrum from 200 to 600 mμ obtained with a Cary recording spectrophotometer. Another portion was dissolved in concentrated sulfuric acid and after two hours the absorption spectrum was determined with the same equipment (26). A final portion was examined by means of a Perkin-Elmer model 21 infrared spectrophotometer, using a scale expander and the potassium bromide pellet method. All spectra were compared with authentic samples from our collection or with published spectra (27).

RESULTS

Quantitative 17-ketosteroid and corticosteroid excretion

In Case 1, the 17-ketosteroid excretion during treatment showed a fourfold increase over the control value, to a level of 23.1 mg. per twenty-four hours. The corticoid excretion during treatment averaged 5.6 mg. per twenty-four hours, which was higher than the control value of 2.9 mg., but still within our range of normal (2 to 6 mg.). In Case 2 the drug was administered for one hundred and two days, but no change was seen except for a single, abrupt rise in the 17-ketosteroid output to 33.7 mg. per twenty-four hours on the twenty-fifth day of treatment, accompanied by a corticoid excretion of 1.5 mg. These values were preceded by a 24-hour excretion of 13.5 mg. of 17-ketosteroids on the fourteenth day of treatment, and were followed by an output of 11.0 mg. on the thirty-third day of treatment. No explanation for this inconsistent behavior can be offered. The quantitative steroid excretion in Case 3 is shown in Figure 1. During both courses of steroid administration there was a significant rise in 17-ketosteroid excretion, without a corresponding increase in excretion of Porter-Silber chromogens. In Case 4 (congenital adrenal hyperplasia), cessation of steroid suppression therapy resulted in a pronounced rise of 17-ketosteroid excretion and also in a definite increase of corticoid excretion to a maximum of 9 mg. per twenty-four hours. The latter value is well above our normal range for urinary Porter-Silber chromogen excretion and suggests, since the disease was not self-limiting at this point, that these chromogens must have had an abnormally low ACTH-suppressant action. The administration of 17α-hydroxyprogesterone did not inhibit the expected rise in urinary steroids following discontinuation of therapy.

Excretion pattern of individual steroids

Our studies of the urinary corticosteroid metabolites were limited to determination of total Porter-Silber chromogens in the various urines, and no attempt was made to isolate the compounds of this type. The Porter-

Silber chromogen values indicate, in fact, that no quantitative change in the excretion of these compounds occurred during the administration of 17α-hydroxyprogesterone.

All the steroids to be discussed were derived from glucuronidase-hydrolysis extracts, since the acid-hydrolysis extracts yielded insignificant amounts of steroid material.

Table 1 lists the compounds which were definitively identified by means of infrared spectra, with additional supporting data from chromatography, melting points, sulfuric-acid chromogen spectra and spot tests. In addition, there were 35 fractions in which adequate purity and definitive identification could not be achieved; the available qualitative evidence is shown in Table 3 (Appendix). Some of these unknowns represented insufficient or impure samples of the substances described in Table 1; others were stereoisomers of identified metabolites, and still others did not correspond to any compounds available to us or identifiable from the literature.

Of the 8 possible isomers of pregnanetriol-3,17,20, four (pregnanetriol-α,α,α; pregnanetriol-α,α,β; allopregnanetriol-α,α,α; and allopregnanetriol-β,α,α) were isolated and identified in this study. Pregnanetriol-3α,17α,20α was by far the major component. Of the four possible isomers of 17-hydroxypregnanolone, three (pregnane-3α,17α-diol-20-one; allopregnane-3α, 17α-diol-20-one; and allopregnane-3β,17α-diol-20-one) were isolated. Isoandrosterone as a metabolite of 17α-hydroxyprogesterone was noted for the first time.

In the patient with congenital adrenal hyperplasia (G.S., Case 4) the control urine contained significant quantities of 17-hydroxypregnanolone, three isomers of pregnanetriol, and androstanedione-3,17, as well as elevated levels of etiocholanolone, androsterone and 11β-hydroxyandrosterone. Administration of 17α-hydroxyprogesterone resulted in a urinary excretion pattern characterized by 1) increased excretion and changes in the proportions of 17-hydroxypregnanolone and pregnanetriol isomers, 2) increased excretion of etiocholanolone and androsterone, with a preponderant change in the former, 3) the appearance of isoandrosterone, and 4) the disappearance of androstanedione-3,17 and 11β-hydroxyandrosterone.

No dehydroepiandrosterone, pregnanolone or pregnanediol was identified in any of the extracts. The finding of 17-hydroxypregnanolone as well as pregnanetriol in the control urine in Case 3 was not unexpected; pregnanetriol has previously been found in normal pregnancy urine (28).

DISCUSSION

Early studies on the administration of 17α-hydroxyprogesterone to animals (6) and man (5, 15) revealed an elevation in 17-ketosteroid excretion in most but not all (16, 19) instances. In more detailed studies (15),

TABLE 1. URINARY STEROIDS DEFINITIVELY IDENTIFIED BEFORE AND DURING ADMINISTRATION OF 17α-HYDROXYPROGESTERONE

Values are minimum quantities, corrected for losses from paper chromatography and extraction procedures. When crystalline material was obtained, corrections were applied to the actual weight of the crystal.

No.	Compound	Case 1				Case 2				Case 3						Case 4			
		Control		17-HP* admin.		17-HP* admin.		17-HP* admin.		Control		17-HP* admin.		Control		Control		17-HP* admin.	
		Amt. (mg.)	M.P. (° C.)	Amt. (mg.)	M.P. (° C.)	Amt. (mg.)	M.P. (° C.)	Amt. (mg.)	M.P. (° C.)	Amt. (mg.)	M.P. (° C.)	Amt. (mg.)	M.P. (° C.)	Amt. (mg.)	M.P. (° C.)	Amt. (mg.)	M.P. (° C.)	Amt. (mg.)	M.P. (° C.)
III	Pregnane-3α,17α-diol-20-one (17-hydroxypregnanolone)	—		26.0	196–200	8.0	203–206	3.0	—	1.0	—	—		1.0	197–202	2.0	200–203	4.0	200–203
VIII	Allopregnane-3β,17α-diol-20-one	—		2.5														1.0	
XII	Allopregnane-3α,17α-diol-20-one	—		1.2															
V	Pregnane-3α,17α,20α-triol (pregnanetriol)	—		14.0	231–235	18.0	238	16.0		1.5		6.0	239	1.5	249–253	6.0	233–238	9.0	
IV	Pregnane-3α,17α,20β-triol	—		2.0		—		4.0		—		—		1.0		2.0	189–192	—	
XIV	Allopregnane-3α,17α,20α-triol	—		1.2	217–233	—		—		—		—		—		2.0		—	
X	Allopregnane-3β,17α,20α-triol	—		1.0		3.5		—		—		—		—		—		3.0	
VI	Etiocholane-3α-ol-17-one (etiocholanolone)	1.2		3.0		8.0		8.0	148–151	0.6		3.0		2.0		12.0	147–150	15.0	144–147
XV	Androstane-3α-ol-17-one (androsterone)	1.3		1.2	178–181	4.0		3.0		—		2.0		2.0		3.0		4.0	
XI	Androstane-3β-ol-17-one (epiandrosterone)	—		1.0	173–175	—		—		—		—		—		—		5.0	173–179
	Androstanedione-3,17	—		1.0		—		—		—		—		—		2.0		—	
	Etiocholanedione-3,17	—		1.0		—		—		—		—		—		—		—	
	11β-Hydroxyandrosterone	1.2		—		—		—		—		—		0.6	198–204	4.0		—	
	11-Keto etiocholanolone	0.7		—		0.7		—		—		—		—		—		—	

* 17-Hydroxyprogesterone.

about five times as much increase was found in etiocholanolone as in androsterone excretion. Bongiovanni *et al.* (16) believed that the "pregnanediol" noted by some investigators (15, 18) was largely if not entirely pregnanetriol, and they identified pregnanetriol-3α,17α,20α by its infrared spectrum in one instance. This finding has been amply confirmed (19), and others have identified small amounts of 17α-hydroxypregnanolone (pregnane-17,20-diol-3-one) (15, 17, 18), pregnane-3,17-diol-20-one (19), and

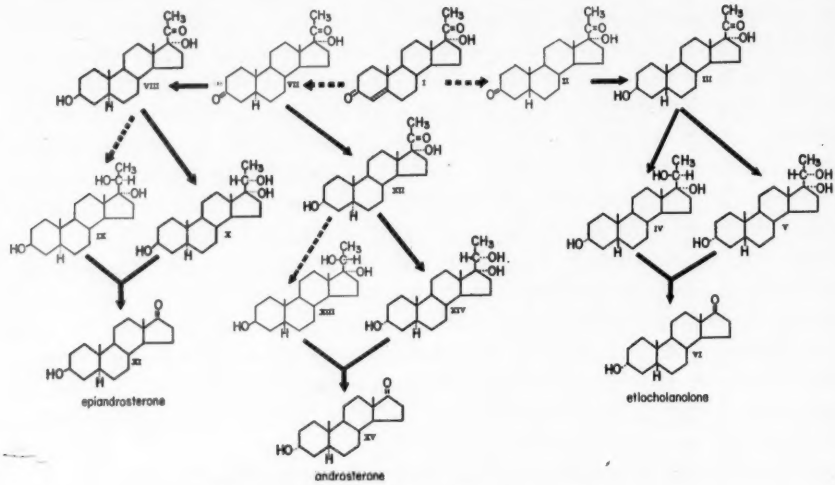

FIG. 2. Metabolism of 17α-hydroxyprogesterone.

pregnane-3α-ol-20-one (15, 18) in acid-hydrolyzed extracts, and even 17α-hydroxyprogesterone (19) when very large doses were given.

The data of Table 1 permit a substantial revision of previously hypothesized pathways (15, 29, 30) for the metabolism of 17α-hydroxyprogesterone (Fig. 2). The major metabolic attack is a full reduction of the Δ^4-3-keto grouping, forming 17α-hydroxypregnanolone and its isomers. Subsequent reduction of the 20-ketone in the side-chain yields pregnanetriol and its isomers. Finally, enzymatic cleavage of the triol side-chain results in the formation of the C_{19} compounds androsterone, etiocholanolone and isoandrosterone. No evidence could be obtained for a reduction of the Δ^4-3-ketone to yield a β-cis configuration at the 3 and 5 positions, which would produce the sequence 3β-17-hydroxypregnanolone$\rightarrow$3β-pregnanetriol$\rightarrow$ etiocholane-3β-ol-17-one.

Dorfman and Ungar (30) postulate the formation of etiocholanedione and androstanedione from 17α-hydroxyprogesterone as the major intermediates in the metabolic pathway by which etiocholanolone and andro-

sterone are formed. Moreover, it is presumed that this is the major route for the formation of these two $C_{19}O_2$ compounds. Minimal amounts of etiocholanedione and androstenedione were found by us in only one instance, and the intermediate compounds pregnane-17α-ol-3, 20-dione and allopregnane-17α-ol-3,20-dione postulated by Dorfman and Ungar were not found in any of the urines.

According to the proposed metabolic scheme, the predominance of etiocholanolone among the excreted $C_{19}O_2$ compounds suggests that the major metabolic pathway is the cleavage of the side-chain of pregnanetriol-3α,17α,20α, and in fact this compound is the major urinary C_{21} metabolite.

Conversely, the small amount of allopregnane-3α-triol would forecast only a small amount of androsterone resulting from side-chain cleavage, and this is also in accord with observed facts. Excretion of androstane-3β-ol-17-one (epiandrosterone) following the administration of 17α-hydroxyprogesterone has been noted for the first time, in 2 of the 4 cases studied. The origin of this material is evident from the presence of the allo-3β isomers of pregnanetriol and 17-hydroxypregnanolone among the urinary metabolites. It is noteworthy that this steroid was observed in the urine of a patient with congenital adrenal hyperplasia as early as 1938 (31).

Other features of androsterone and etiocholanolone excretion are also of interest. It is pertinent that there was adequate thyroid function or replacement therapy in all instances, since it has been shown that the level of thyroid activity affects the ratio of urinary etiocholanolone and androsterone (32). In our patients these two ketosteroids formed a distinctly variable proportion of the total metabolites identified. In Case 1, substantial quantities of pregnanetriol plus 17-hydroxypregnanolone but only small amounts of androsterone and etiocholanolone were produced, whereas in Case 2 the relative output of $C_{19}O_2$ compounds was appreciably higher. The ratio of these two ketosteroids is also subject to individual variation. Although an E/A ratio of 5:1 in 8 patients given 17α-hydroxyprogesterone has been reported (15), in our studies the ratio was not constant and it did not exceed 4:1 in any instance. This emphasizes once again the individual variation in the steroid metabolic pattern and underscores the point that, although predominant excretion of this or that metabolite may be characteristic of a particular steroid, far-reaching deductions from arithmetical devices such as the E/A ratio are hazardous.

Variations in the pattern of excretion of the C_{21} compounds were also outstanding. Overall output ranged from the maximum 18 mg. of total pregnanetriols plus 30 mg. of total 17-hydroxypregnanolones in Case 1, to 4.5 mg. of pregnanetriols and no 17-hydroxypregnanolones in Case 3. In a specific subject, changes in the excretion pattern also occur from time to time, as shown by the quantitative studies in Case 2 and Case 3 (Fig. 1)

and by the comparison of the two urine specimens during treatment in Case 2. Such observations lend added significance to the qualitative and quantitative differences noted by various workers after administration of a particular steroid, as for example, testosterone (33) or 21-desoxyhydrocortisone (15, 17, 34–36). Methodology has generally been thought to account for these differences and the possible role of individual variation has been tacitly minimized. Our findings suggest the need for caution in making quantitative generalizations from data obtained in small numbers of cases.

No pregnanolone was observed in any of the urines analyzed. Reports of the presence of this compound (15, 17) have followed the use of alumina chromatography, occasionally preceded by mineral-acid hydrolysis. These conditions are known to favor 17-hydroxyl removal (36), and the possibility of artefact formation must be kept in mind.

There is now considerable information available regarding the urinary steroid pattern in congenital adrenal hyperplasia on the one hand, and of the extra-adrenal metabolism of the intermediates in adrenal corticosteroid synthesis on the other. It should be possible, therefore, to specify the particular enzymatic "bottleneck" in adrenal hormone synthesis from a study of the urinary metabolites, as shown in Table 2.

In the event of inadequate 21-hydroxylation with high activity of the other (11- and 17-hydroxylating) systems, an increased output of 21-des-

TABLE 2. POSSIBLE BIOSYNTHETIC DEFECTS IN ADRENOCORTICAL STEROIDOGENESIS

The minus signs do not indicate *total* absence of a particular hydroxylase system.

Steroid-oxygenating activity at positions			Major adrenal steroid secreted	Major known or potential urinary metabolites	Clinical syndrome
17	11	21			
+	+	+	Cortisol	Tetrahydrocortisol, etc.	Normal
+	+	−	21-Desoxycortisol	11-Oxypregnanetriols; 11-oxy-17-hydroxypregnanolones	Congenital adrenal hyperplasia with 11-ketotriols (28, 40)
+	−	+	Reichstein's S	Tetrahydro "S" and "S"-3,17, 20,21-tetrol	Hypertensive congenital adrenal hyperplasia (41)
−	+	+	Corticosterone	Tetrahydro-"B" and "B"-3,11, 20,21-tetrol	Normal in part. Total absence of 17-hydroxylation unknown
+	−	−	17-Hydroxyprogesterone	Pregnanetriols; 17-hydroxy-pregnanolones	Congenital adrenal hyperplasia (15, 16, 42)
−	+	−	(11β-Hydroxyprogesterone)	Pregnane-3,11,20-triols; pregnane-3,11-diol-20-ones	Unknown
−	−	+	(Desoxycorticosterone)	Tetrahydro-desoxycorticosterone; pregnane-3,20,21-triols	Unknown
−	−	−	(Progesterone)	Pregnanediols; pregnanolones	Unknown

oxyhydrocortisone would be expected. The preponderant metabolites of this compound appear to be the 11-oxygenated derivatives of 17-hydroxypregnanolone and pregnanetriol (with the 11-keto type predominating in one study (35), and approximately equal quantities of the 11-keto and 11-hydroxy in another (34)). Since the 11-oxygen function, once in place, is not removed (37), side-chain cleavage results chiefly in $C_{19}O_3$ derivatives. These substances comprise from less than 1 per cent (35) up to 6 per cent (34) of the total metabolites. The proportions of the 11-hydroxy and 11-keto derivatives of androsterone and etiocholanolone varied greatly from case to case, without the consistent predominance of the 5β (etio) configuration noted in the metabolism of 17α-hydroxyprogesterone. Examples of congenital adrenal hyperplasia with urinary steroid excretion resembling the pattern seen after administration of 21-desoxy hydrocortisone have been published (38, 39).

In the event that both 21- and 11-oxygenation are faulty, adrenocortical biosynthesis would tend to produce excess amounts of 17α-hydroxyprogesterone. It is now clear that extra-adrenal metabolism of this steroid leads to increased excretion of several pregnanetriols (chiefly the α,α,α-isomer) and of 17-hydroxypregnanolones (also primarily in the α,α configuration). Further metabolic alteration leads to the production of etiocholanolone and lesser amounts of androsterone and isoandrosterone. Such a urinary excretion pattern resembles that seen in most instances of congenital adrenal hyperplasia (15, 16, 41). However, significant differences, particularly with regard to the excretion of $C_{19}O_2$ and $C_{19}O_3$ metabolites have been noted (41). Although we do not attach great significance to the numerical value of the E/A ratio, it is true that the relatively increased androsterone excretion in congenital adrenal hyperplasia requires explanation, as does the relatively large output of 11-hydroxyandrosterone compared to that of 11-keto-etiocholanolone. It is known that metabolism of administered dehydroisoandrosterone, Δ^4-androstenedione and 11β-hydroxy-Δ^4-androstenedione yields metabolites preponderantly of the 5α (andro) configuration. It has been suggested (13), therefore, that these compounds, individually or collectively, comprise the "adrenal androgen" which is secreted in excess as a result of continuous stimulation by ACTH. Indeed, incubation of radioacetate with adrenal tissue from a patient with congenital adrenal hyperplasia yielded relatively little radioactive 17α-hydroxyprogesterone and considerable amounts of dehydroisoandrosterone, Δ^4-androstenedione and 11β-hydroxy-Δ^4-androstenedione in the ratio of 17:1:3 (42). The high proportion of the Δ^5 compound indicates that the majority of the androgens are not produced by side-chain cleavage of 17α-hydroxyprogesterone. Thus there is an excellent possibility that hyper-

APPENDIX

TABLE 3. CHARACTERISTICS OF STEROID FRACTIONS NOT DEFINITIVELY IDENTIFIED

Abbreviations for chromatographic systems are: B = benzene; Cy = cyclohexene; F = formamide; MeC = methyl cyclohexane; MC = methylene dichloride; PG = propylene glycol; T = toluene. Fluorescence was developed with fuming sulfuric acid treatment and excited with an ultraviolet source.

Unknown No.	Chromatographic system	Conc. H_2SO_4 spectrum λ_{max} (O.D.)	Major infrared max. ($cm.^{-1}$)	M.P. (°C.)	Comments
Case 1. Control					
X-1	B-F 18 hrs.	263 (0.6); 282 (0.99); 362 (0.3)	—	—	Blue fluorescence
X-2	MeC-PG 72 hrs.	313 (0.9); 450 (0.75)	—	—	Pink fluor.; 17-keto (Zimmermann positive)
X-3	MeC-PG 72 hrs.	318	—	—	Light green fluor.
X-4	MeC-PG 96 hrs.	310	1275, 1080, 1055–1060, 990, 940, 840	—	Orange fluor.
X-5	MeC-PG 14 days	307 (1.0); 400 (0.3)	—	—	17-Keto (Zimm. pos.)
X-6	MeC-PG 60 hrs.	264 (1.45); 312 (1.5); 410 (0.3)	—	—	Peach fluor.; 17-keto (Zimm. pos.)
X-7	MeC-PG 18 hrs.	294 (2.15); 397 (0.45); 390 (0.1)	diketone	—	17-Keto (Zimm. pos.)
X-8	MeC-PG 50 hrs.	312 (1.0); 335 (0.9); 405 (0.25)	diketone	—	17-Keto (Zimm. pos.)
Case 1. 17-OH-progesterone administration					
X-9	MC-F 3 hrs.	255 (1.87); 290 (0.43)	1727–1737, 1595, 770, 710	238–240	Blue fluor.
X-10	MC-F 3½ hrs.	262 (1.3); 290 (0.7)	1725–1739, 1265–1270, 1080, 910, 868, 775	—	17 Keto (Zimm. pos.); blue fluor.
X-11	Cy-F 10 hrs.	306 (1.57); 412 (0.5)	20-ketone, 3-hydroxy	—	Isomer of C_{21} diol monoketone
X-12	Cy-F 10 days	312 (0.65); 430 (0.3); 500 (0.16)	prēgnanetriol isomer	—	Orange fluor.
X-13	Cy-F 26 hrs.	308 (0.55); 375 (0.30)	—	—	Deep pink-orange fluor.
Case 2. First 17-OH-progesterone administration					
X-15	Cy-F 12 hrs.	310 (0.95); 380 (0.88)	1763, 1362, 1100–1110, 945, 880, 850, 770–775	—	Pink-orange fluor.
X-16	T-PG 48 hrs.	307 (1.2); 450–455 (1.15)	—	—	λ_{max}(alc) 280 mμ; green-yellow fluor.
X-17	MC-F 10 hrs.	235 (2.2); 308 (1.35)	1665	—	Blue fluor.; λ_{max}(alc) 292 mμ
X-18	MeC-PG 24 hrs.	312 (1.0); 410–415 (0.25)	1735	—	17-Keto (Zimm. pos.)

(*Continued*)

X-19	T-PG 7 days	275 (0.85); 305 (0.5); 375 (0.4)	1080, 1035, 920, 850, 760	—	17-Keto (Zimm. pos.)
X-20	MeC-PG 48 hrs.	305 (1.53); 372 (1.6)	1085, 1045, 980, 925, 850, 775	—	17-Keto (Zimm. pos.)
Case 3. Control					
X-23	MeC-PG 72 hrs.	308 (0.68); 437 (0.98); 500 (0.2)	20-hydroxy, 3-hydroxy	—	Pregnanetriol isomer
X-24	Cy-F 12 hrs.	314 (0.85); 400 (0.4); 495 (0.18)	20-ketone, 3-hydroxy	—	17-Hydroxypregnanolone isomer
X-25	MeC-PG 10 hrs.	312 (1.6); 345 (1.55); 425 (0.6)	—	—	Orange fluor.
Case 3. 17-OH-progesterone administration					
X-26	Cy-F 15 hrs.	262 (0.7); 302 (0.33)	1695–1700, 1655–1657, 1595, 850, 755, 742	—	λ_{max}(alc) 272 mμ.
X-27	Cy-F 4 hrs.	338 (0.75); 387 (0.71); 433 (0.72)	1735–1737	—	Orange fluor.
Case 3. Interim control					
X-21	MeC-PG 72 hrs.	302 (1.4); 395 (1.07); 440 (1.4)	—	—	Orange fluor.; + with Pb tetra-acetate
X-22	MeC-PG 18 hrs.	297–300 (1.93); 400 (0.55); 490 (0.18)	1315, 1265, 1125, 1105, 1050	—	17-Keto (Zimm. pos.)
Case 4. Control					
X-28	Cy-F 14 hrs.	260 (2.2); 325–330 (0.6)	—	—	Blue fluor.; λ_{max}(alc.) 232, 297 mμ.
X-29	Cy-F 20 hrs.	313	1735, 1460, 1375, 1160, 1005–1010, 950, 875, 835, 795, 720	—	Pink fluor.
X-30	Cy-F 20 hrs.	310 (0.75); 435 (0.48); 500 (0.2)	3, 20-dihydroxy	—	Pregnanetriol isomer
X-32	MeC-PG 4 days	302 (1.0); 372 (0.43); 399 (0.40)	1725–1730, 1700, 1055, 1070, 1015, 960, 940, 910, 875	—	17-Keto (Zimm. pos.)
X-31	MeC-PG 9 days	312 (1.25); 400 (0.6)	—	—	17-Keto (Zimm. pos.)
X-33	MeC-PG 4 days	312 (1.9); 334 (1.65); 402 (0.5)	—	—	17-Keto (Zimm. pos.)
Case 4. 17-OH-progesterone administration					
X-34	Cy-F 16 hrs.	312 (0.6); 375 (0.28); 490 (0.1)	—	183–188	Pink fluor.
X-35	MeC-PG	310 (0.9); 334 (0.92); 380	—	—	17-Keto (Zimm. pos.)
X-14	MeC-PG 12 hrs.	297 (1.8); 405 (0.5)	1730–1735, 1703–1708	146–152	17-Keto (Zimm. pos.)

function of a distinct synthetic pathway, rather than the intra-adrenal or extra-adrenal metabolism of excess 21-desoxyhydrocortisone or 17α-hydroxyprogesterone, accounts for the androgenic manifestations associated with this disease.

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DIURNAL VARIATION OF 17-HYDROXYCORTICOSTEROIDS, SODIUM, POTASSIUM, MAGNESIUM AND CREATININE IN NORMAL SUBJECTS AND IN CASES OF TREATED ADRENAL INSUFFICIENCY AND CUSHING'S SYNDROME*†

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ABSTRACT

The morning rise and the nocturnal drop in plasma and urinary levels of 17-hydroxycorticosteroids (17-OH-CS) and in urinary sodium, potassium and endogenous creatinine clearance are described in 8 normal subjects receiving a controlled diet composed of equal feedings every three hours. Magnesium excretion was highest during the period of lowest sodium and potassium excretion (3 A.M. to 6 A.M.). Endogenous creatinine clearance had two peaks (6 P.M. to 9 P.M. and 6 A.M. to 9 A.M.) with two low periods (noon to 3 P.M. and 2 A.M. to 6 A.M.). A definite increase in both sodium and potassium excretion was noted during oral administration of graded dosages of cortisol hemisuccinate in patients with treated adrenal insufficiency. Rapid intravenous injection of 5 mg. of cortisol hemisuccinate every three hours into similar subjects obliterated the variations in creatinine clearance but failed to influence decisively the nocturnal drop in sodium, potassium and magnesium excretion. Magnesium excretion was not influenced by administration of cortisol in either group. Five subjects with Cushing's syndrome due to bilateral adrenal hyperplasia had constant levels of 17-OH-CS in plasma and urine throughout the twenty-four hours. These 17-OH-CS values were most strikingly abnormal when compared to normal nocturnal values. The disturbed rhythm in 17-OH-CS levels in these patients was associated with a disturbance in sodium and potassium excretion, but the noctural excretion peak for magnesium was normal.

A MARKED decrease in sodium and potassium excretion at night has been demonstrated by many investigators (1–4). This nocturnal decrease persists whether the electrolyte intake occurs during the day or is

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distributed evenly throughout the twenty-four hours (3, 4). Reversal of day-night activity does not result in prompt reversal of the excretory pattern of sodium and potassium. The first reversal of electrolyte excretion in a normal subject was reported by Norn in 1929; and the time required was six weeks (2). In another investigation, it was claimed that the reversal could occur with greater rapidity if the subject slept during the day and looked directly at a light during the night (5). It was suggested that a rhythmical secretion of adrenal hormones of the 17-hydroxycorticosteroid (17-OH-CS) type could account for the day-night difference in the excretions of these cations (5). This hypothesis became more attractive with the demonstration that a definite diurnal variation in the plasma level (6–8) and in the urinary excretion of 17-OH-CS (7, 9) existed. The main objection to this hypothesis was that adrenocortical hormones have generally been considered to cause a decrease in sodium excretion and an increase in potassium excretion (3), whereas sodium and potassium normally vary in the same direction at any given time in the daily cycle.

In 1929 Norn (2) studied the diurnal variation in magnesium excretion in humans. He found an increased secretion of magnesium at night. No diurnal variation was found in calcium excretion.

The object of the present investigation was to determine whether the 24-hour variations in sodium, potassium and magnesium excretion are caused by a rhythmical secretion of 17-OH-CS.

MATERIALS AND METHODS

Eight normal subjects were given exactly 180 ml. of milk and 2 large graham crackers every three hours for twenty-seven hours. Plasma and urine samples were collected every three hours during this period and analyzed for 17-OH-CS, sodium, potassium, magnesium and creatinine. All subjects studied, whether normal or abnormal, spent the time from 0630 (6:30 A.M.) to 2100 (9:00 P.M.) sitting, standing or walking and were recumbent from hours 2100 to 0630.

Studies on subjects with adrenal insufficiency were divided into three groups according to the following schedule of cortisol dosage. Plasma and urine specimens were collected as in the normal series.

Time	*Group I*	*Group II*	*Group III*
900 (9:00 A.M.)	5 mg.	0 mg.	2.5 mg.
1200 (noon)	5 mg.	0 mg.	5 mg.
1500 (3:00 P.M.)	5 mg.	2.5 mg.	10 mg.
1800 (6:00 P.M.)	5 mg.	5 mg.	10 mg.
2100 (9:00 P.M.)	5 mg.	10 mg.	5 mg.
2400 (midnight)	5 mg.	10 mg.	2.5 mg.
300 (3:00 A.M.)	5 mg.	5 mg.	0 mg.
600 (6:00 A.M.)	5 mg.	2.5 mg.	2.5 mg.

The 7 subjects in Group I were given cortisol hemisuccinate intravenously. The 3 subjects in Group II and the 3 subjects in Group III were given cortisol hemisuccinate

orally. The schedule of dosage resulted in maximal intake of cortisol from hours 2100 (9 P.M.) to midnight in Group II and from hours 1500 (3 P.M.) to 1800 (6 P.M.) in Group III. All subjects with adrenal insufficiency were well compensated and had been treated with an average of 25 mg. of cortisol daily and 0.2 mg. of 9α-fluorohydrocortisone daily for three months to two years before the study.

The normal changes in plasma cortisol concentration might have been simulated more closely by the use of a continuous intravenous infusion of cortisol in the adrenal insufficiency groups. However, it would have resulted in decreased activity and interference with the normal postural changes of the subjects. This would introduce another variable into the comparisons with the normal group, since posture and activity are known to have a definite influence on renal function and electrolyte excretion (10–12).

Five patients with Cushing's syndrome were studied under conditions identical to those for the normal subjects.

Plasma and urinary 17-OH-CS were measured by the Peterson *et al.* modification (13) of the method of Silber and Porter (14). Sodium and potassium were measured on the Baird internal standard flame photometer. Magnesium was measured by Garner's (15) modification of the titan yellow method of Kolthoff. Creatinine was measured by the method of Hare and Hare (16) with adsorption onto Lloyd's reagent to eliminate nonspecific chromogens.

RESULTS

The normal 24-hour variation in the levels of plasma and urinary 17-OH-CS is shown in Figure 1. Plasma 17-OH-CS concentration underwent a striking rise from a mean low of 1.9 μg. per 100 ml. at midnight to a mean high of 13.6 μg. per 100 ml. at hour 0900 (9 a.m.). The variations in urinary 17-OH-CS followed the plasma variations closely but were of lesser magnitude. The lag between peak plasma and peak urinary 17-OH-CS levels was three hours or less.

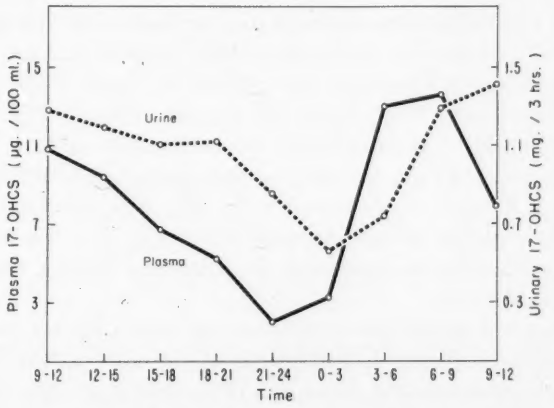

FIG. 1. Mean spontaneous variation in plasma and urinary 17-OH-CS in 8 normal subjects. Individual variations did not always coincide with the mean, but peak high and low values were rarely displaced more than three hours from the mean.

The electrolyte excretory pattern for these normal subjects is shown in Figure 2. The lowest excretion of sodium and potassium occurred in the three-hour period following the lowest excretion of 17-OH-CS. After reaching a nighttime low of 6 mEq. per three hours, sodium excretion increased to 12.4 mEq. per three hours and potassium excretion increased from 3.5

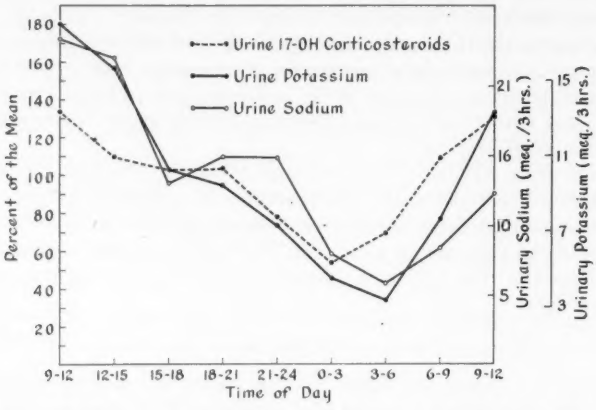

FIG. 2. Demonstration of the relationship of the mean diurnal variation in urinary sodium and potassium to 17-OH-CS excretion in 8 normal subjects. The lowest electrolyte excretion occurred in the three-hour period following the lowest excretion of 17-OH-CS. Mean urinary three-hour excretions were: 17-OH-CS, 1.1 mg; sodium, 14.0 mEq.; and potassium, 10.3 mEq.

mEq. to 13.7 mEq. per three hours. Endogenous creatinine clearance (Fig. 3) exhibited a bimodal curve with the high periods occurring at hours 1800–2100 (130 ml. per minute) and hours 0600 to 0900 (122 ml. per minute). The corresponding low periods were present at hours 1200–1500 (112 ml. per minute) and hours 0300–0600 (105 ml. per minute). The drop in clearance from 130 ml. per minute to 105 ml. per minute and the rise from 105 ml. per minute to 122 ml. per minute were statistically significant, with a P value of $<.01$ and $<.05$ respectively (17). The rise from 112 ml. per minute to 130 ml. per minute became significant ($P<.05$) when our total experience with subjects receiving this diet and having normal adrenal function was analyzed.

In Figure 4 are shown the corresponding values for the adrenal insufficiency patients of Group I. Flattening of the adrenal excretory rhythm curve did not obliterate the day-night difference in sodium and potassium excretion. Although the magnitude of the day-night excretion differences for sodium and potassium was slightly diminished, the change was not significant. Endogenous creatinine clearance measurements in this group of

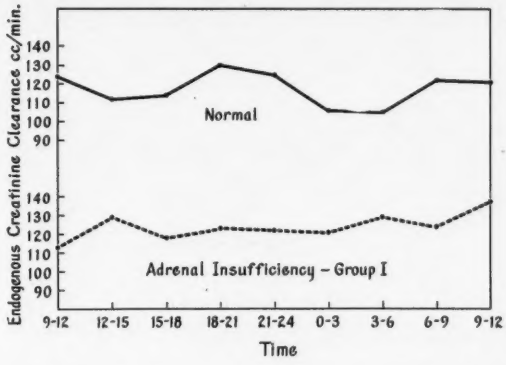

FIG. 3. Elimination of the normal bimodal peak in endogenous creatinine clearance (corrected to 1.73 sq.M. of body surface) by intravenous injection of 5 mg. of cortisol hemisuccinate every three hours in patients with adrenal insufficiency (Group I).

subjects (Fig. 3) failed to reveal the 24-hour variation seen in normal subjects. Instead, the clearance values underwent a gradual rise throughout the period of study.

The values for the adrenal insufficiency patients in Group II are shown in Figure 5. Urinary 17-OH-CS values were within the normal range but the maximum excretion occurred six hours earlier than normal. The sodium

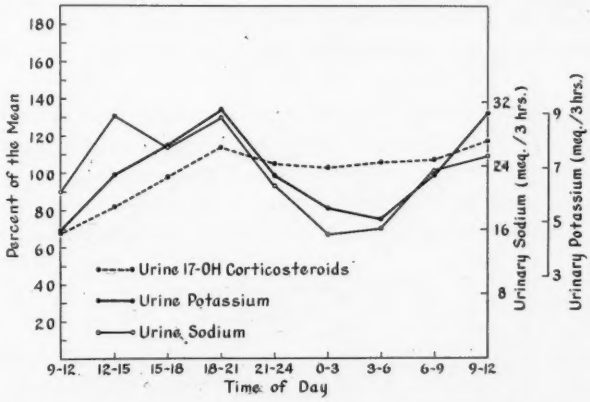

FIG. 4. Urinary excretion of sodium, potassium and 17-OH-CS in 7 patients with adrenal insufficiency (Group I) given intravenous injections of 5 mg. of cortisol hemisuccinate every three hours. Note eventual flattening of 17-OH-CS excretion curve without a corresponding change in the electrolyte excretion curves. Mean urinary three-hour excretions were: 17-OH-CS, 1.43 mg.; sodium, 22.9 mEq.; and potassium 6.8 mEq.

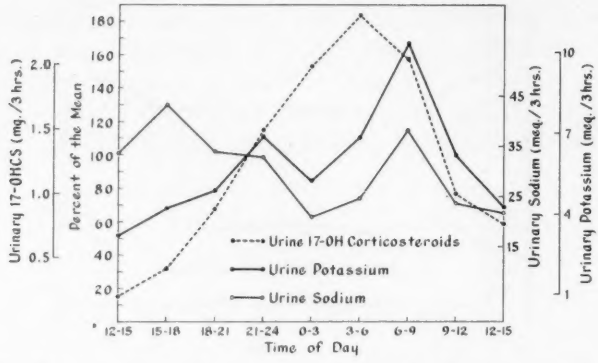

FIG. 5. Simultaneous increase in urinary sodium and potassium excretion associated with graded doses of oral cortisol hemisuccinate administered to 3 patients with adrenal insufficiency (Group II). The 17-OH-CS excretion peak was reached six hours earlier than normal, as was the peak for electrolyte excretion. Mean urinary three-hour excretions were: 17-OH-CS, 1.3 mg.; sodium, 34.3 mEq.; and potassium 6.2 mEq.

and potassium excretion peaks also occurred about six hours earlier than normal. In Group III the peak excretion of 17-OH-CS was observed twelve hours earlier than normal (Fig. 6) and caused still earlier excretion peaks of sodium and potassium. In all groups with adrenal insufficiency, the mean sodium excretion was greater and the mean potassium excretion was somewhat less than in the normal group.

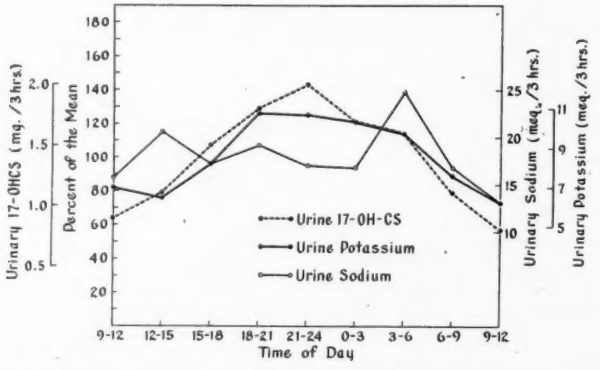

FIG. 6. Excretion curves in 3 patients with adrenal insufficiency (Group III), given graded doses of cortisol hemisuccinate orally so that the 17-OH-CS excretion peak occurred twelve hours earlier than normal. Although the electrolyte excretion peaks are not as distinct, they occurred still earlier than in the patients of Figure 5. Mean urinary three-hour excretions were: 17-OH-CS, 1.4 mg.; sodium, 17.9 mEq.; and potassium, 8.5 mEq.

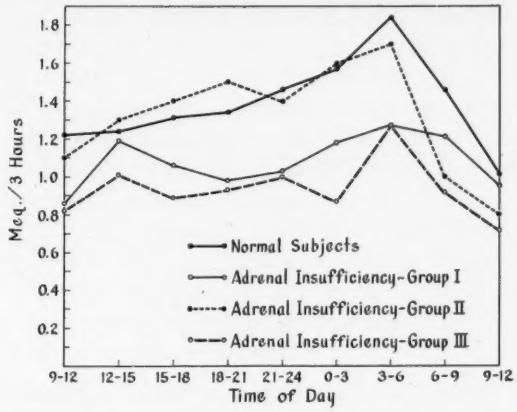

FIG. 7. Mean magnesium excretion over twenty-seven hours in all subjects was maximal from hours 0300 to 0600 (3:00 to 6:00 a.m.) regardless of the 17-OH-CS excretory pattern.

Magnesium excretion was also measured simultaneously in all of these subjects (Fig. 7). The normal subjects exhibited a peak excretion of magnesium during the night (hours 0300–0600) at a time when excretion of sodium and potassium was at its lowest. Creation of abnormal 17-OH-CS excretion peaks in the patients with adrenal insufficiency (Groups I–III) failed to alter the excretion peak of magnesium. The major excretion of magnesium occurred during the hours 0300–0600 for all groups studied. Urinary calcium excretion was also determined, but no diurnal variation was found.

Figure 8 shows the plasma and urinary 17-OH-CS values obtained in 5 patients with Cushing's syndrome due to bilateral adrenal hyperplasia. The striking nocturnal drop in corticoid values seen in the normal subjects was completely absent. The plasma levels averaged 27 μg. per 100 ml. This was close to the upper limit of normal for morning levels (normal range, 4–26

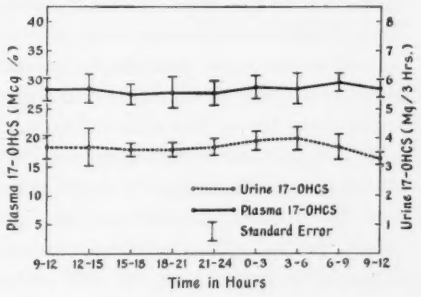

FIG. 8. Values for plasma and urinary 17-OH-CS in 5 patients with Cushing's syndrome due to bilateral adrenal hyperplasia. These patients did not exhibit the diurnal variation noted in normal subjects.

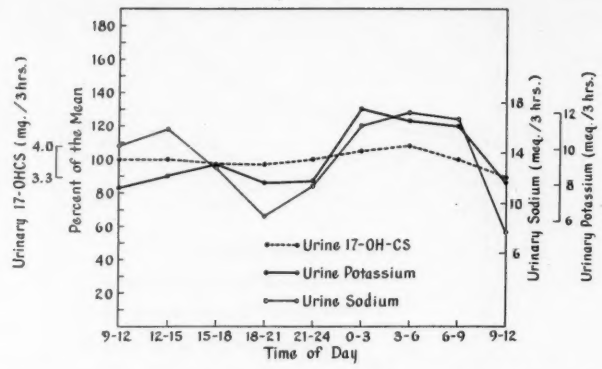

FIG. 9. Relationship of urinary 17-OH-CS to sodium and potassium excretion in 5 patients with Cushing's syndrome. The highest excretion of these electrolytes occurred during a period when electrolyte excretion was low in normal subjects.

μg. per 100 ml.), but was greatly elevated when compared to normal night values (normal, 0–6 μg. per 100 ml. at midnight). Although the mean urinary excretion of 3.7 mg. per three-hour period was elevated for all times of the day, it was especially elevated when compared to the mean nocturnal excretion in normal persons. Figure 3 shows the mean electrolyte excretion figures in these subjects. The diminution in sodium and potassium excretion during the hours from 0300 to 0600 not only failed to take place, but an actual nocturnal diuresis occurred (Fig. 9). The significance of the nocturnal rise in sodium and potassium excretion cannot be evaluated statistically because of the smallness of the group, but the change was consistently present. The pattern of magnesium excretion was normal in this group.

DISCUSSION

These studies define the relationships between the diurnal variations in plasma and urinary 17-OH-CS and urinary sodium, potassium, magnesium and endogenous creatinine clearance in normal subjects. The magnitude of variation in plasma 17-OH-CS levels was greater than the urinary changes. This might be expected, since the urinary values probably reflect an average of the various plasma values present during the three-hour collection period. The use of six-hour and eight-hour collection periods, as in previous studies by the authors and others, can obscure certain changes, since high and low plasma values would sometimes be included in a single period. A marked rise in plasma 17-OH-CS concentration occurs before the onset of the day's physical activity, as noted previously (4). Therefore this rise might be thought of as preparation for the activity to come, rather than as a response to it. In all but one instance the individual peaks in plasma 17-

OH-CS concentration correlated well with the subject's opinion regarding the time of day of his greatest efficiency. The single exception was a well established "late starter" whose plasma 17-OH-CS peak occurred at 6 A.M. Establishment of the existence of a diurnal variation in mental and physical efficiency has been described by others (18). Patients with untreated adrenal insufficiency are known to have abnormal electroencephalographic patterns (19) which revert towards normal during glucocorticoid therapy. Thorn and associates (20) noted a striking improvement of mental efficiency in patients with adrenal insufficiency during treatment with cortisone acetate. A relationship between glucocorticoid activity and mental activity would therefore seem established in specific situations. It is possible that the diurnal variation in adrenal 17-OH-CS might be a major factor in determining the period of greatest mental efficiency in the 24-hour period. Means of controlling this peak efficiency period would have practical application in industries employing night workers, and in the armed services. This would be especially true for the Air Force with its recurrent need for night flights during which a slight improvement in mental efficiency might be of great importance. Attempts to reverse the adrenal rhythm by a reversal of activity have been reported as unsuccessful in night workers (21), with a more recent report of success after more complete activity reversal (22).

A definite increase in both sodium and potassium excretion was noted after the highest doses of cortisol in adrenal insufficiency Groups II and III. The increase in potassium excretion after cortisol was not unexpected. However, an increase in sodium excretion after administration of cortisol is not consistent with the sodium retention and edema formation noted during treatment of various clinical states. An increase in sodium excretion has been noted after administration of glucocorticoid in specific situations (23–25). In general, our experience is similar to that of Laidlaw (26) who noted that a dosage of cortisol below 5 mg. per hour increased sodium excretion whereas a dosage above 10 mg. per hour decreased it. The reason for this is not clear. It has been postulated that at low dosage levels the increase in cortisol causes a proportionately greater increase in the filtration than in the reabsorption of sodium (27). At higher dosage levels the cortisol effect on reabsorption becomes more important and sodium is retained.

The reappearance of a normal sodium and potassium rhythm was noted in adrenal insufficiency Group I. A factor, in addition to cortisol, which influenced sodium and potassium excretion must have been present. It is unlikely that this factor was aldosterone, since the patients had primary adrenal insufficiency. Moreover the reported nocturnal decrease in aldosterone excretion in normal subjects would tend to increase sodium excretion at night rather than decrease it (28, 29). No consistent relationship

was noted between endogenous creatinine clearance and sodium and potassium excretion. A change in filtration by the kidney probably does not offer a satisfactory explanation. This finding must remain unexplained at present. A search for an extrarenal substance which has a rhythmical influence on tubular function, in addition to cortisol and aldosterone, would seem to have some justification.

Sirota, Baldwin and Villarreal (30) investigated diurnal variations in glomerular filtration, using creatinine clearance and inulin clearance simultaneously. No difference was noted between creatinine and inulin clearance. These workers did not find a bimodal peak in endogenous creatinine clearance as was found in our study, but a tendency to such a pattern can be observed on inspection of their data. Perhaps the use of four-hour collection periods obscured the magnitude of the variations.

Addis and associates (31) concluded that diurnal variations in creatinine clearance were not due to variations in protein intake. Our study confirms that finding, since the diurnal variation persisted in the normal subjects when the protein intake was divided evenly over the twenty-four hours.

A definite diurnal variation was found in the excretion of magnesium. The inverse relationship of magnesium excretion to sodium and potassium excretion was probably not significant, since alteration of excretion of the latter electrolytes in adrenal insufficiency Groups II and III was not accompanied by a change in magnesium excretion. Suggestive evidence of an effect of adrenocorticotropin (ACTH) on magnesium excretion has been reported by others (32). The present study shows that at low levels of cortisol administration, there is no discernible effect on magnesium excretion. This does not rule out the possibility of an effect at higher levels of cortisol induced by stimulation with ACTH. The lack of effect on magnesium excretion noted in the present study is of some interest because of the definite effect of cortisol on the other major intracellular cation—potassium. Control of the diurnal variation of magnesium excretion appears to be relatively independent of the mechanisms for control of sodium and potassium excretory rhythms.

Perhaps the most interesting finding in this study was the complete lack of variation in plasma and urine 17-OH-CS levels in 5 patients with Cushing's syndrome (Fig. 8). Absence of a urinary diurnal variation has been referred to previously by others (33, 34), but no actual data were given. The patients in the present study all had bilateral adrenal hyperplasia, proved subsequently at the time of operation. The free plasma 17-OH-CS levels were not elevated above the normal range when measured at the conventional forenoon times. However, when compared to those in normal subjects at night, the levels were ten to fifteen times normal. The urinary

values were elevated for all periods of the day and night, but again showed the most abnormality when compared to normal night values. It may be that the most characteristic and damaging feature of Cushing's syndrome occurs during the night hours when exceedingly low values are found in normal subjects. Since completing this study we have studied a patient with Cushing's syndrome due to bilateral hyperplasia who exhibited a normal rhythm in both plasma and urinary 17-OH-CS values. Again, however, his plasma levels were normal in the forenoon hours, but abnormal when compared to normal nocturnal plasma levels. His urinary excretion of 17-OH-CS was also more strikingly abnormal when evaluated at night than by day. It would appear that the accuracy of the diagnosis of Cushing's syndrome may be improved in some cases by a careful examination of night plasma and urine 17-OH-CS values. It is our current practice to determine 9 a.m. and 9 p.m. plasma 17-OH-CS levels on all subjects suspected of having Cushing's syndrome.

The nocturnal increase in sodium and potassium excretion noted in the patients with Cushing's syndrome has been described by others in patients with congestive heart failure and cirrhosis with ascites (5, 35). It may be a phenomenon common to all sodium-retention states. Elimination of postural changes by having the subjects assume the same position day and night is said to eliminate the disturbance in sodium excretion and to permit the appearance of a normal diurnal rhythm in congestive heart failure (5). No such studies have been performed to date in Cushing's syndrome. The change in sodium and potassium excretion was not accompanied by a change in magnesium excretion in these patients. This evidence further supports the conclusion that magnesium excretion is probably controlled by an independent mechanism.

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PUBERAL MATURATION OF THE NORMAL HUMAN TESTIS. A HISTOLOGIC STUDY

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ABSTRACT

A histologic study was made of testicular biopsy specimens obtained from 13 normal youths 9 to 16 years old, in order to determine the pattern of the maturation which takes place in the normal testis during the period ranging from prepuberal age until adulthood. Observations on the tubular content, tubular wall and intertubular connective tissue, indicated three degrees of maturation: 1) the beginning of puberal development, 2) an intermediate degree between the puberal and adult structure, and 3) testes which have practically reached adulthood. In testes showing the first degree of maturation, three stages of lobular evolution were observed. Organization of the tubular wall became manifest in two well differentiated zones, *i.e.*, basal membrane and tunica propria. A definite sequence was observed in the development of the three component parts: amorphous substance, fibers and cells. In the course of testicular maturation the amorphous substance was replaced by reticular fibers and, at the same time, fibroblasts showed signs of maturation. In the intertubular connective tissue, transition was observed from inactive prepuberal tissue to active embryonic tissue, and subsequently to an intertubular connective tissue similar to that of an adult testis. These changes occurred in the cellular elements as well as in the amorphous substance and fibers. These findings, besides demonstrating the histologic characteristics of the process of testicular maturation during normal puberty, should serve as a pattern with which to compare histologically abnormal testes, particularly when the condition may be due to abnormal maturation during puberty.

THE histologic morphology of normal testes during puberty has been a matter of consideration on the part of numerous investigators (1–5).

In the present paper we attempt to demonstrate the histologic changes which take place in the normal human testis from prepuberal age until adulthood, *i.e.*, the transition from a prepuberal testis to an adult testis, and to establish the sequence in which these gradual changes occur. It is

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hoped that it will facilitate the histologic interpretation of pathologic testicular patterns. Particular attention is paid to the following points:

1. The gradual steps of testicular maturation.
2. Development of the connective tissue (cells, fibers and amorphous substance) of the tubular wall and intertubular spaces, with special reference to the origin and fate of the Leydig cells.
3. Interrelation between the development of the tubular content, tubular wall and intertubular connective tissue.

MATERIAL AND METHODS

Our investigations were carried out on 13 normal puberal boys whose ages, at the time the biopsies were performed, are shown in Table 1. No effort was made to establish a relationship between the chronologic age and the histologic findings. Somatically, these boys exhibited various physiologic degrees of puberal development.

Testicular biopsies were performed according to the technic described by Charny (6), and histologic studies were carried out as follows:

a) for general morphology: hematoxylin-eosin and Azan trichromatic stains.
b) for cytologic morphology: Del Rio Hortega's panoptic silver impregnation method.
c) for collagen and reticular fibers: Del Rio Hortega's silver impregnation technic.
d) for elastic fibers: orcein stain.
e) for histochemical technics, see Mancini *et al.* (7)

RESULTS

Our presentation refers mainly to histologic findings, with special reference to morphology. The histochemical results are also included, when necessary for the sake of clarity. Special attention has been given to the connective tissue rather than to the tubular content.

The hormonal and clinical findings were similar to those described by other authors for male puberty (8).

Figure 1 is a graphic representation of the progressive degrees of histologic maturation found in the testes under investigation. For didactic purposes and based upon the histologic characteristics of the tubular content, tubular wall and intertubular connective tissue, the puberal testes were classified in three groups:

1. First degree of maturation (Cases 1, 2, 3 and 4).
2. Second degree of maturation (Cases 5, 6 and 7).
3. Third degree of maturation (Cases 8, 9, 10, 11, 12 and 13).

This classification allows better appreciation of the morphologic changes which gradually take place after the prepuberal age and before the testes reach full maturity (Fig. 1).

During the first-degree period of maturation certain fundamental changes were observed which denote what may be termed the beginning of

the histologic puberty of the testis. Therefore, special attention was concentrated on this initial degree of testicular maturity. Examination of serial sections showed three different stages of progressive maturation in the testicular lobules, as indicated in Figure 1. This subdivision was made only in the first-degree maturation group, and was considered necessary for a better understanding of the evolutive changes which take place during this particular period.

The differentiation between "degrees of maturation" and "lobular

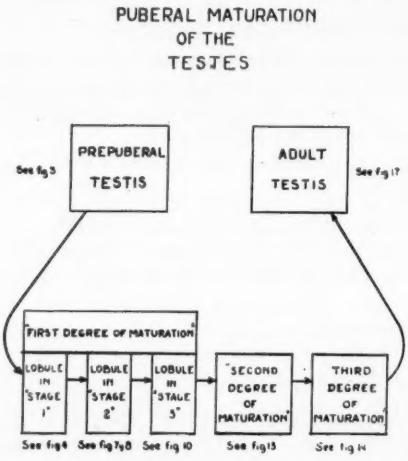

FIG. 1. Sequence of normal maturation of the human testis from prepuberal age to adulthood.

stages" should be borne continually in mind in order to facilitate the interpretation of what otherwise would appear to be a very complex paper.

The morphologic characteristics of the tubular content, the tubular wall and the intertubular connective tissue are described separately.

The morphologic characteristics of the different cellular types found in the connective tissue are depicted in Figure 2, including the abbreviations used in nomenclature.

Testes in first degree of maturation

The process of maturation starts at the periphery of the lobules and progresses gradually towards the center. At the same time, vascular congestion appears in the albuginea and septa.

The progressive development of the testicular lobules during the period of first-degree maturation was classified in "stages" according to the histologic features. Lobules in stage 1 were the least mature; lobules in stage 3 were the most mature; and lobules in stage 2 represented an intermediate evolution between the previous two.

TABLE 1. COMPARISON BETWEEN CHRONOLOGIC AGE AND HISTOLOGIC MATURATION OF TESTIS

Case No.	Age of boy	Histologic maturation of testis
1	11 y. 8 m.	First degree of maturation
2	10 y.	First degree of maturation
3	10 y. 8 m.	First degree of maturation
4	9 y. 3 m.	First degree of maturation
5	14 y. 11 m.	Second degree of maturation
6	13 y. 11 m.	Second degree of maturation
7	12 y. 6 m.	Second degree of maturation
8	12 y. 10 m.	Third degree of maturation
9	13 y. 10 m.	Third degree of maturation
10	16 y. 9 m.	Third degree of maturation
11	12 y. 5 m.	Third degree of maturation
12	15 y. 11 m.	Third degree of maturation
13	16 y.	Third degree of maturation

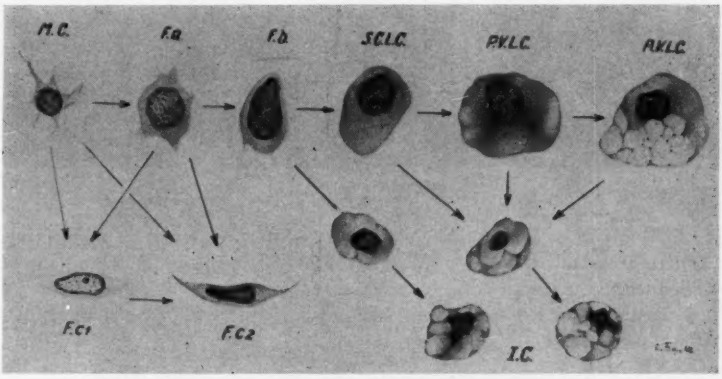

FIG. 2. Morphology and probable evolution of the different cellular types found in the intertubular tissue and tubular wall. Probable course of evolution indicated by arrows.

Key to abbreviations: M.C. = mesenchyma-like cells. F.a = type "a" fibroblasts. F.b = type "b" fibroblasts. F.c.1. and F.c.2 = subdivisions of type "c" fibroblasts. S.C.L.C. = small and compact Leydig cell. P.V.L.C. = poorly vacuolated Leydig cell. R.V.L.C. = richly vacuolated Leydig cell. I.C. = "involutive" cell; to the left is the cell which originates in type "b" fibroblasts; to the right is the cell which originates in Leydig cells.

Lobules in stage 1. These do not differ much from the lobules in prepuberal testes (Fig. 3), except for vascular congestion of the albuginea and septa; initial maturation occurs in the parenchyma in the outer part of the lobule (upper left, Fig. 4). The tubules are of small size and rather scarce, being separated from each other by a mesenchyma-like tissue (lower center, Fig. 4).

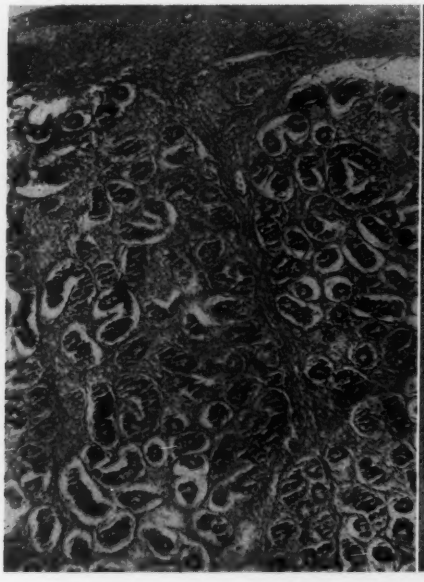

FIG. 3

FIG. 4

FIG. 3. Testis of a 6-year old child. Lobules are separated by connective tissue septa. There are small tubules with undifferentiated epithelial cells and intertubular spaces filled with immature fibroblasts. (Hematoxylin-eosin ×62.)

FIG. 4. Testis showing first degree of maturation. Panoramic view of lobule in stage 1. (Hematoxylin-eosin ×62.)

The tubular content (Fig. 5) consists of undifferentiated epithelial cells.

The tubular wall (Fig. 5) is arranged in band-like homogeneous tissue, there being no indication, as yet, of tunica propria or basal membrane. Two types of fibroblasts—"a" and "c"—are present, the former being more numerous (Figs. 5 and 2). These fibroblasts have a large nucleus, loose chromatin granules and a thin nuclear membrane; no nucleolus can be seen. Cytoplasm is scanty and a few elongations can be detected by means of the panoptic method. Types "a" and "c" can be found in the external part of the tubular wall, but in the internal portion there is a predominance of type "c" (Fig. 2). The last named have the same character-

istics as those present in adult connective tissue. Two morphologic varieties—"c.1" and "c.2"—are discernible; both are elongated and have no cytoplasmatic extensions. Type "c.1" (Fig. 2) has a nucleus with loose chromatin and a large nucleolus; it occurs in larger numbers than type "c.2" (Fig. 2), which has an oval nucleus with dense chromatin and no nucleolus.

The intertubular connective tissue (Figs. 4, 5 and 6) is quite abundant, and its structure resembles that of mesenchymal tissue. The vessels are collapsed. Four kinds of cells are identifiable: a) mesenchyma-like, resembling juvenile fibroblasts of the embryonic mesenchyma (Figs. 6 and 2) and having a small nucleus with pale chromatin, scarce cytoplasm, and multiple elongations which appear to be interconnected; b) type "a" fibro-

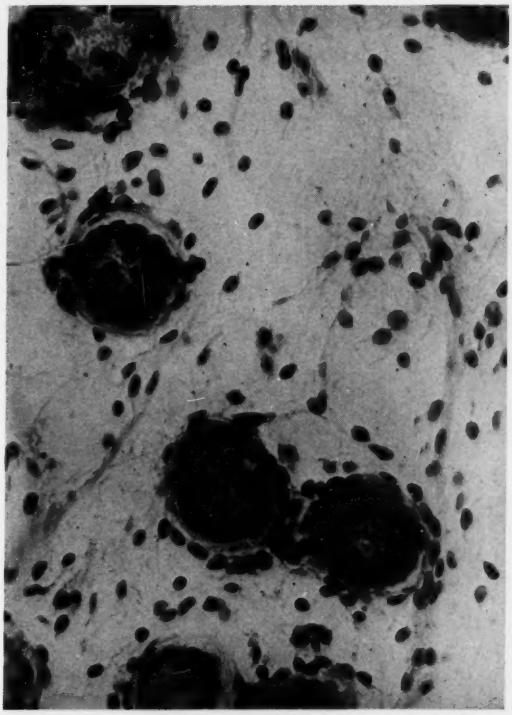

FIG. 5. Testis—first degree of maturation. Lobule in stage 1. Tubular content formed by undifferentiated cells. No differentiation between basal membrane and tunica propria. Tubular wall contains types "a" and "c" fibroblasts, the former on the outside and the latter on the inside. Intertubular connective tissue is slightly fibrillar, and contains mesenchyma-like cells and fibroblasts of the three types ("a," "b" and "c"). (Hematoxylin-eosin ×420.)

blasts in considerable numbers; c) type "c" fibroblasts in abundance; and d) type "b" fibroblasts (Fig. 2) in small numbers; these have a smaller nucleus than type "a," but dense chromatin and a nucleolus; the cytoplasm tends to adopt a round shape, and with the panoptic technic very fine elongations therefrom can sometimes be observed.

Lobules in stage 2. These show progressive steps of maturation, intermediate between those of stage 1 (Fig. 4) and stage 3 (Fig. 10). The main difference when compared with lobules in stage 1 is that the mesenchymal structures have continued their maturation towards the central part (Figs. 7 and 8).

In the corresponding intertubular connective tissue (Fig. 9) two striking features are apparent: 1) congested and dilated blood vessels, and an abundant substance—hereafter referred to as "plasma-like"—with the same histologic and staining characteristics as that of the plasma contained in the neighboring blood vessels, and 2) a moderate number of type "b" fibro-

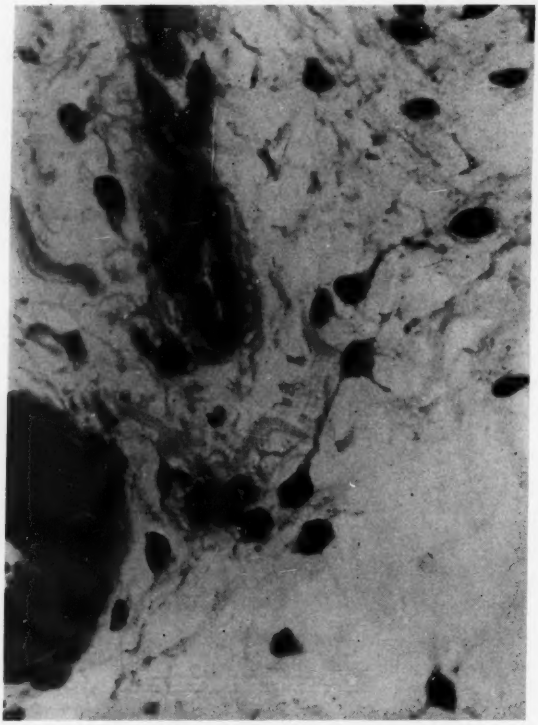

Fig. 6. Testis—first degree of maturation. Lobule in stage 1. Intertubular connective tissue contains mesenchyma-like cells. (Hematoxylin-eosin ×940.)

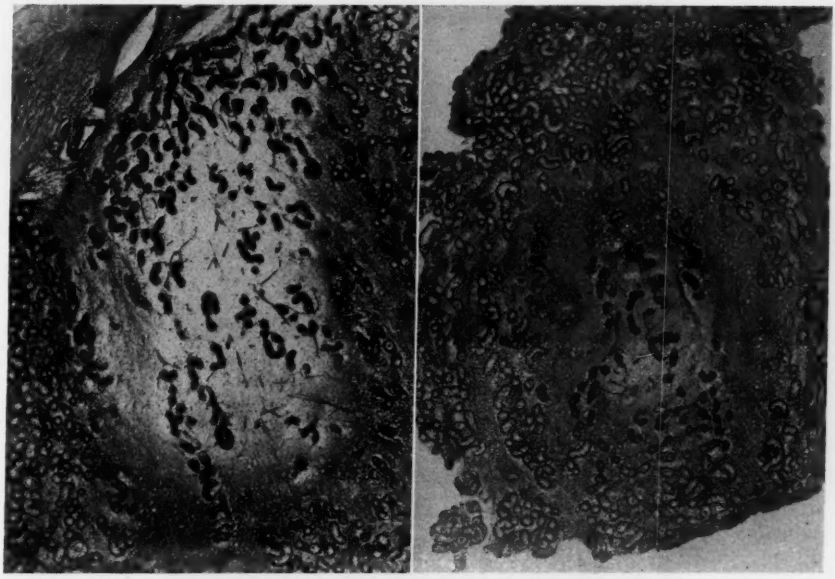

FIG. 7 FIG. 8

FIGS. 7 and 8. Testes showing first degree of maturation. Lobules in stage 2. Low magnification in order to show progressive maturation from stage 1 to stage 3. Maturation progresses inward from the periphery of the lobule. Figure 8 (central portion) shows a small area where maturation has not yet started. (Hematoxylin-eosin ×27.)

blasts. A great number of these cells show positive reactions for lipids, ascorbic acid and nucleoproteins in the cytoplasm and (as discussed later) seem to be precursors of the type of Leydig cells which we have described as "small and compact." The mesenchyma-like cells have disappeared at this stage of testicular development. Instead, types "a" fibroblasts are present in moderate quantity, and type "c" in very small quantity.

Lobules in stage 3. This is the most advanced stage in the first degree of maturation of the human testis (Fig. 1). Vascular congestion has decreased, and the tubules appear more closely packed and their diameter is larger (Fig. 10).

As regards the tubular content (Fig. 11), the corresponding cells are more numerous and some spermatogonia and primary spermatocytes are present. Sertoli cells are seen in small quantities.

When the tubular wall (Fig. 11) is compared with that in lobules in stage 1, it appears to be thinner and two distinct zones are evident: an internal one which is homogeneous and represents the basal membrane, and an external one which is fibrillar and corresponds to the tunica pro-

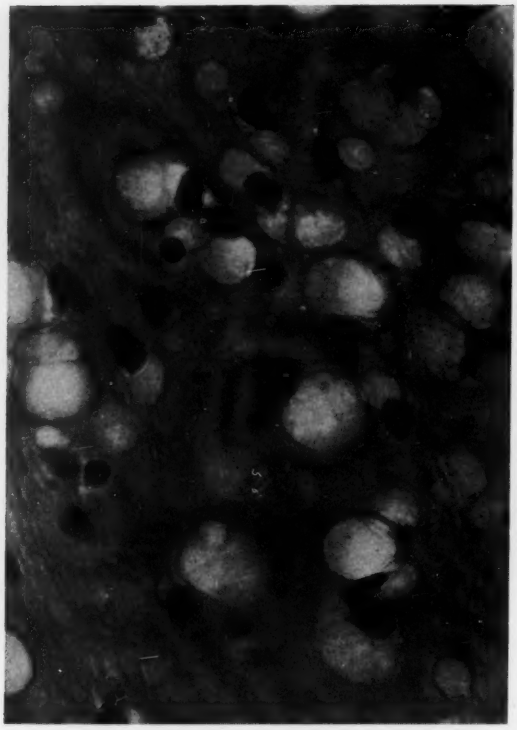

FIG. 9. Testis—first degree of maturation. Lobule in stage 2. Intertubular spaces are filled with abundant plasma-like substance of homogeneous appearance. Types "a" and "b" fibroblasts are predominant. (Hematoxylin-eosin ×900.)

pria. In the latter there are abundant type "c.1" fibroblasts; type "c.2" fibroblasts are adjacent to the basal membrane. Type "a" fibroblasts can be observed in the outer aspect of the tunica propria.

The intertubular connective tissue (Fig. 11) appears to be less widely distributed than in lobules in stage 1. The plasma-like substance is less abundant and more fibrillar in appearance, and the number of reticular fibers is increased. An abundance of type "b" fibroblasts can be observed. However, there is a decrease in the number of types "a" and "c" fibroblasts. A new variety of cell, which will be referred to as "involutive" (Figs. 12 and 2), has now appeared. These cells have a pyknotic nucleus, abundant cytoplasm and numerous vacuoles, but neither crystals nor pigment are discernible. Between type "b" fibroblasts and the "involutive" cells, there are various transitional types (Figs. 12 and 2). Type "c" fibroblasts gradually exhibit more cytoplasm; vacuolation develops, and the

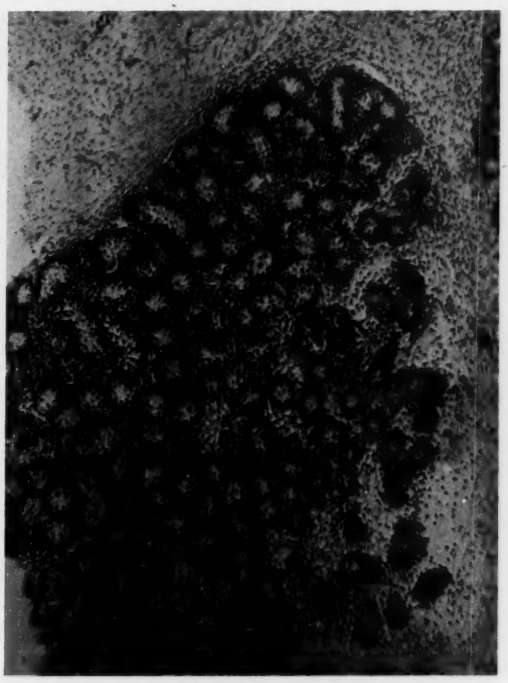

FIG. 10. Testis—first degree of maturation. Panoramic view of lobule in stage 3. (Hematoxylin-eosin ×80.)

chromatin becomes more and more condensed in the nucleus. Mast cells and plasma cells are also apparent in the intertubular connective tissue.

Testes in second degree of maturation

Lobules are larger and tubules have also increased in diameter, but intertubular spaces have become smaller (Fig. 13). At this juncture in development, lobules often show areas with transitional steps between second and first, and second and third degrees of maturation.

Examination of the tubular contents shows that the germinal epithelium now contains spermatids and, occasionally, spermatozoa. At the same time one can observe Sertoli cells in a well advanced stage of evolution.

The tubular wall is thicker and the delimitation of basal membrane and tunica propria is more precise. Types "c.1" and "c.2" fibroblasts are present in the outer and inner zones of the tubular wall, but in lesser quantities than in the prior degree of maturation. Type "a" fibroblasts are located in the outer zone of the tunica propria.

In the intertubular connective tissue it is now possible to discern a va-

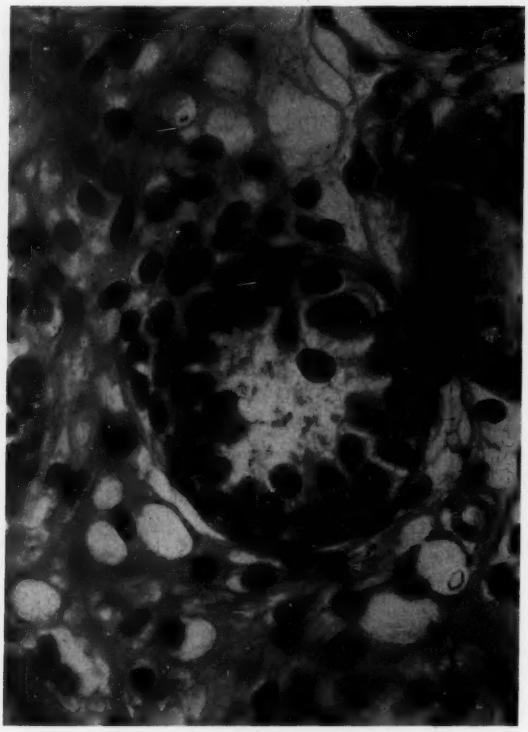

FIG. 11. Testis—first degree of maturation. Lobule in stage 3. Seminiferous tubule with some spermatogonia and occasional Sertoli cells. Tunica propria is becoming manifest and contains type "c.1" fibroblasts; type "c.2" are adjacent to the basal membrane. The intertubular connective tissue shows a moderate amount of plasma-like substance as well as types "a" and "b" fibroblasts. (Hematoxylin-eosin ×800).

riety of Leydig cell. This cell, however, is smaller and more compact than its adult prototype and hereafter will be identified by the salient characteristics, "small and compact" (Fig. 2). In comparison with type "b" fibroblasts, these cells have a polygonal or rounded contour, their cytoplasm is more abundant and their granules are finer. They also have a smaller nucleus, a thicker membrane, dense granules of chromatin and a large nucleolus. Moreover, they are eosinophilic and are not vacuolated.

Type "b" fibroblasts and "involutive" cells are abundant, and type "c" fibroblasts are more numerous than in the first degree of maturation. Only a few type "a" fibroblasts can be seen.

The plasma-like substance is less abundant and in some areas only reticular fibers can be observed.

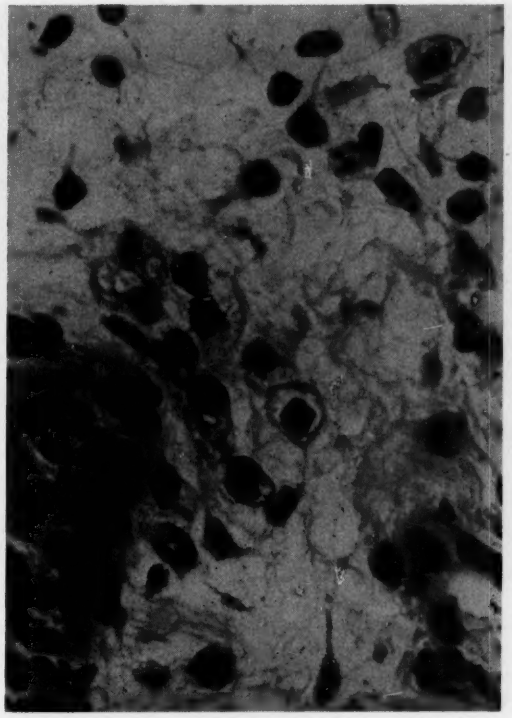

FIG. 12. Testis—first degree of maturation. Lobule in stage 3. Group of "involutive" cells in different transitional stages starting from type "b" fibroblasts. (Hematoxylin-eosin ×940.)

Testes in third degree of maturation

In these testes (Fig. 14), which are very similar to those of normal adult persons, the lobules have continued increasing in size, and the tubules are of a larger diameter.

Typical tubular contents are shown in Figures 14 and 15. Spermatogonia, spermatids and spermatozoa are present in varying amounts, and Sertoli cells are more numerous.

Compared with the histologic picture in the first degree of maturation, the tubular wall (Figs. 14, 15 and 16) has become thicker in spite of the greater diameter of the tubules. The tunica propria and the basal membrane are now clearly differentiated and, from a morphologic point of view, they are similar to those of the adult testis. Type "c" fibroblasts are present in moderate quantities, and subgroup "c.2" cells have localized predominantly in the basal membrane. Type "c.1" fibroblasts continue to be pres-

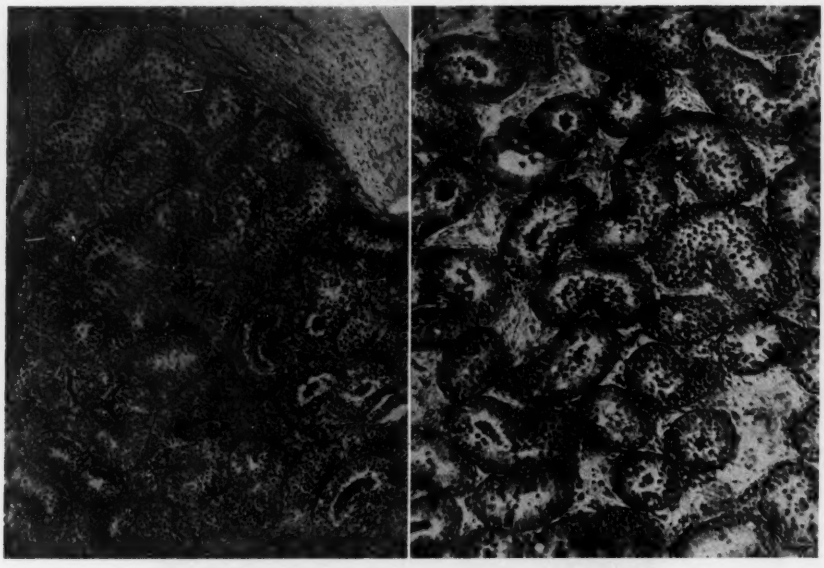

FIG. 13 FIG. 14

FIG. 13. Testis—second degree of maturation. Tubules have reached medium size—increased diameter and more advanced development of the tubular wall and germinal epithelium. However, spermatogenesis is still incomplete. Intertubular connective tissue shows less plasma-like substance and the cells are less numerous. A few "small and compact" Leydig cells are now apparent. (Hematoxylin-eosin ×62.)

FIG. 14. Testis—third degree of maturation. Tubules have increased in size. Spermatids and a few spermatozoa are seen. The tunica propria is well developed. The intertubular connective tissue contains abundant fibroblasts and Leydig cells, both "small and compact" and "vacuolated." (Hematoxylin-eosin ×62.)

ent in the tunica propria, and a small number of type "a" cells persist in the most external part of the tubular wall. Elastic fibers have also appeared in the tubular wall but, when compared with those of adult testes, they are less numerous, thinner and stain less distinctly.

In the intertubular connective tissue (Figs. 15 and 16) the spaces are smaller than those in testes showing the first and second degrees of maturation, the plasma-like substance persisting only in a few areas. Pericellular nets of reticular fibers are greater in number and there is a moderate quantity of intercellular fibers. The total number of cells is smaller. Type "a" fibroblasts appear to be fewer, whereas type "c" are more abundant. Type "c.1" fibroblasts differ histochemically from those of testes in the first and second degrees of maturation, since they react positively for lipids and alkaline phosphatase. Numerous "small and compact" Leydig cells can be

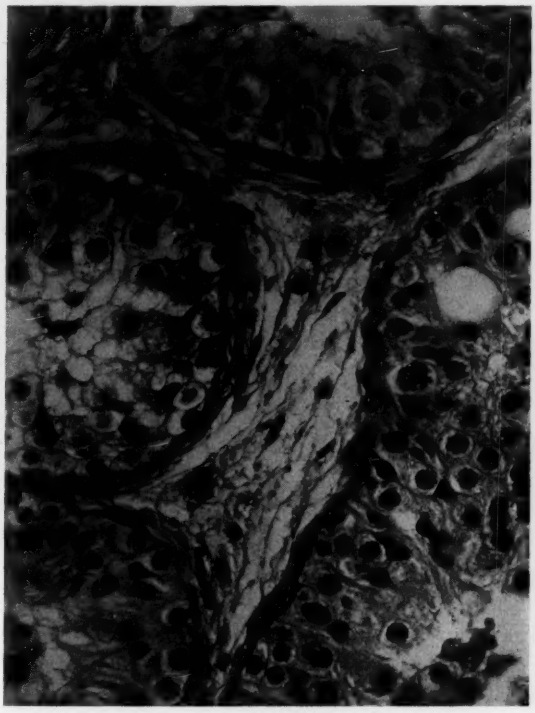

FIG 15. Testis—third degree of maturation. Germinal epithelium with spermatids, primary spermatocytes and Sertoli cells. Well developed basal membrane and tunica propria, the former containing type "c.2" fibroblasts. The intertubular connective tissue is of fibrillar stucture, and contains Leydig cells (both "small and compact" and "vacuolated") and type "c.2" fibroblasts. (Hematoxylin-eosin ×420.)

observed, surrounded by nets of reticular fibers. However, two other types of Leydig cells have now appeared in abundance; they are both vacuolated, but one more distinctly than the other, for which reason we have identified them as "poorly vacuolated" and "richly vacuolated" Leydig cells. The former (Figs. 16 and 2) has a polyhedral contour and is larger in size than the "small and compact" Leydig cell. Its cytoplasm is abundant and intensely eosinophilic in the perinuclear area. Its nucleus is bounded by a thick membrane, and its vacuoles are arranged peripherally. The bundles of reticular fibers which surround these cells are well developed. The second type of vacuolated Leydig cell (Figs. 16 and 2) is somewhat larger than the one just described and the vacuolation of its cytoplasm extends further into the peripheral area.

"Involutive" cells have increased in number (Fig. 16). Between these

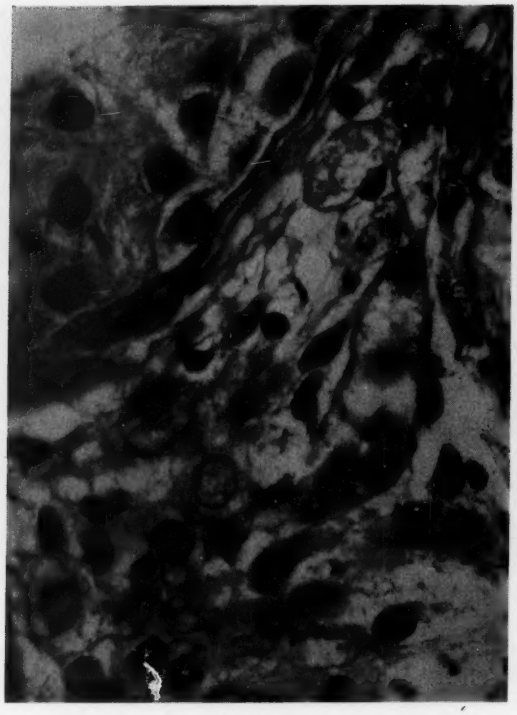

FIG. 16. Testis—third degree of maturation. In the tubular wall the basal membrane is clearly differentiated from the tunica propria which contains type "c.1" fibroblasts. The intertubular spaces show type "b" fibroblasts, "poorly" and "richly" vacuolated Leydig cells, and cells in stages of transition between them and the "involutive" cells. (Hematoxylin-eosin ×940.)

cells and the different types of Leydig cells—"small and compact" and "vacuolated"—numerous transitional stages are evident, apparently the result of a process of cytoplasmatic vacuolation, reduction in the cellular volume and nuclear pyknosis (Figs. 16 and 2).

Neither crystals nor pigment can be observed in any of the three types of Leydig cells, nor in the "involutive" cells.

Adult testis

In this degree of maturation the three fundamental structures of the testis (Figs. 17 and 1) can be considered fully developed.

Inasmuch as numerous investigators, including ourselves (7, 9) have already described the histologic characteristics of the normal adult human testis, we refrain from doing so in this paper.

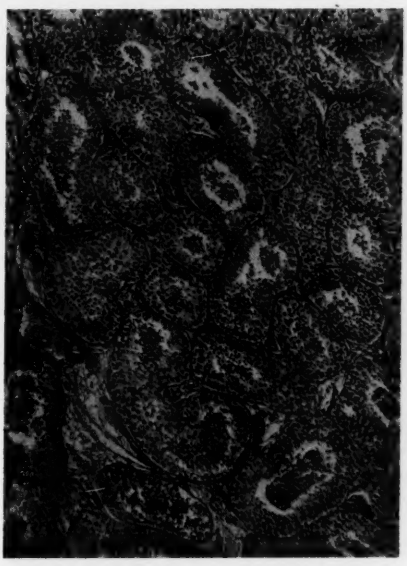

FIG. 17. Adult human testis.
(Hematoxylin-eosin ×62.)

DISCUSSION

During puberty there is a close relationship between the development of primary and secondary sexual characteristics on the one hand, and the histologic maturation of the testes, on the other. However, as is well known, the latter does not run parallel with the chronologic age of the subject.

The progressive histologic changes which take place in the three fundamental structures of the human testis (Fig. 1) lead naturally to a classification of first, second and third degrees of maturation.

The first degree of maturation corresponds to the beginning of puberal development, whereas the third degree indicates a testis which has practically reached adulthood.

The first degree of maturation include testes with lobules in three stages of evolution: 1) lobules similar to those of prepuberal testes, 2) lobules showing progressive degrees of intermediate maturation, and 3) lobules similar to those in testes showing the second degree of maturation.

It is our impression that lobules in stage 1 of the first degree of maturation indicate the beginning of testicular pubescence. This is based upon our observations (the subject of a subsequent publication) on testes from birth up to the seventh year of age, during which period of time the histologic picture remains practically unchanged.

Testicular maturation reflects itself in each individual lobule from pre-

puberta! age to adulthood. However, these gradual changes do not take place in all lobules simultaneously; thus one particular human puberal testis may contain lobules in different stages of development.

Tubular content

The germinal epithelium first becomes manifest in the stage-2 lobules of the first degree of maturation; the adult pattern is reached in the course of the second and third degrees of maturation towards adulthood.

It is in stage 3 of the first degree of maturation that Sertoli cells first appear, their adult features becoming manifest in the third degree of maturation.

Tubular wall

As puberal maturity progresses, the tubular wall changes from a band-like homogeneous tissue to a structure containing two well differentiated zones, *i.e.*, basal membrane and tunica propria.

The cells in the tubular wall vary in type according to the degree of maturation of the puberal testis. Before the tubular wall becomes organized, there is a predominance of type "a" fibroblasts, and only in the inner aspect of the wall can a few more mature type "c" fibroblasts be observed. When the tunica propria has acquired its adult characteristics, the final arrangement of the fibroblasts from the outside inward is as follows: juvenile (type "a"), intermediate (type "c.1") and mature (type "c.2").

This transformation in the fibroblasts of the tubular wall, as well as the presence of lipids and alkaline phosphatase, lends support to our belief that they play an important role in the development and organization of the tubular wall (10) in the course of testicular maturation.

It is very important to establish whether the activity of these fibroblasts is regulated: a) by androgens produced by the Leydig cells, b) by the hypophyseal gonadotropins, or c) by some other stimulus which may originate in the intratubular cells. During our investigation of testes showing the second degree of maturation we noted that the tubules with "small and compact" Leydig cells in their vicinity were the ones with a more fully developed wall. Williams (11) described Leydig cells in the proximity of the tubules of grafted testes in adult rabbits; the tubules underwent fibrosis when the interstitial cells also disappeared from the graft. The observations of Sobel *et al.* (12) in boys with pseudo-puberty suggest that androgens may participate in the development of the tubules. All these facts lend support to the theory that the development and/or maintenance of the tubular wall is influenced in a manner as yet undetermined, by androgenic activity.

As we have shown previously (13), elastic fibers are found in the tubular wall of testes in the third degree of maturation.

Intertubular connective tissue

In the first degree of maturation, there is a striking difference between the lobules in stages 1 and 2. In the former, the connective tissue is mainly of the embryonic type—mesenchyma-like cells and type "a" fibroblasts—and contains collapsed blood vessels. In the latter, however, there is proliferation of juvenile fibroblasts (types "a" and "c.1") and type "b" fibroblasts, associated with dilated and congested blood vessels. Intertubular spaces are filled with a plasma-like substance. These manifestations may be due to the action of hypophyseal gonadotropins. In this connection, it should be remembered that Hartman *et al.* (14) have shown that administration of chorionic gonadotropin induces an intense vascular dilatation in rat testes.

During the whole cycle of maturation of the testes it is observed that the plasma-like substance gradually diminishes, while an increase occurs in the number of reticular fibers, first around types "a" and "b" fibroblasts, and later around the different types of Leydig cells.

We have also observed a change in the proportion of different types of cells in the connective tissue. It would appear that the so-called mesenchymal cells are the precursors of fibroblasts and Leydig cells, as shown in detail in Figure 2. Mesenchyma-like cells were seen only in testes in the first degree of maturation; although they were abundant in stage 1 of lobular development, they had disappeared in stage 3. Type "a" fibroblasts were also abundant in the first degree of maturation but diminished gradually with the oncoming of adulthood. Type "b" fibroblasts were moderately abundant in lobules corresponding to stage 2 of the first degree of maturation, but became very numerous in stage 3. From then on, they were exceeded in number by the "small and compact" Leydig cells in testes in the second degree of maturation. We believe that type "b" fibroblasts are the precursors of these Leydig cells, since we have detected numerous individual cells showing histologic signs of transition between these two types. Sniffen (3) and Albert and Underdahl (4) believe that Leydig cells originate in mesenchymal cells morphologically similar to fibroblasts. Similar observations have been recorded by Hooker (15) for bulls' puberal testes, and by Williams (11) for grafted adult rabbits' testes.

The "small and compact" Leydig cells were observed in the second and third degrees of maturation. They differ from those found in adult testes (2, 7), in that they are arranged in small groups, their size is smaller, their contour is less clearly defined, and their nucleus and nuclear membrane stain with less precision.

There is a tendency to believe that, at puberty, Leydig cells appear later than spermatozoa (1, 2, 4, 5). However, in testes showing the first degree of maturation we have noted the appearance of type "b" fibroblasts simultaneously with the beginning of differentiation of the germinal epithelium. "Small and compact" Leydig cells were observed by us from then on, in testes in the second and third degrees of maturation, and the vacuolated types of Leydig cells were present only in the last or third degree of maturation. There seems to be some relationship between these findings and those reported by Hooker (15) in bulls' puberal testes.

It was noted that the Leydig cells finally underwent involution, thus confirming Hooker's findings in bovine testes (15), as well as the observations of Sniffen (3) in adult human testes.

The abundant "involutive" cells which appeared in the stage-3 lobules of testes in the first degree of maturation may perhaps be indicative of failure, on the part of intertubular connective tissue cells of puberal testes, to respond to a continuous and sustained stimulation.

The maturation of the human testis during puberty is partly the result of specific hormonal stimuli (*e.g.*, gonadotropin and thyroid hormone) which are capable of inducing, either directly or indirectly, the modifications which take place in the cellular and intercellular elements of the connective tissue of the tubular wall and intertubular spaces. The connective tissue in the tunica albuginea also shows cellular proliferation, vascular congestion and edema during puberal maturation (16).

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HISTOLOGIC STUDY OF THE UNDESCENDED HUMAN TESTIS DURING PUBERTY

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ABSTRACT

A histologic study was made of puberal unilateral undescended testes in 7 boys, selected according to the following criteria: a) that they were in the puberal age group, 2) that their unilateral cryptorchism was due to mechanical obstruction, and 3) that a control biopsy be carried out simultaneously on the scrotal testis as well as on the undescended testes of the same patient. The results were compared with the findings in a group of normal puberal testes. Changes were observed in the three fundamental components of the testis, *viz*, the tubular content, the tubular wall and the intertubular connective tissue. These changes were classified in two groups which were designated: 1) delayed normal maturation, and 2) pathologic maturation. The former manifested itself by smaller tubules and decreased cellular maturation of the germinal epithelium. In the intertubular connective tissue such delay was specially observed in the maturation of the cellular population. Pathologic maturation showed up as morphologic abnormalities in the cells of the germinal epithelium. In the connective tissue there was proliferation of fibroblastic and collagen fibers in the intertubular tissue and tubular wall, and the wall had acquired greater thickness. There was an increase in the number of "involutive" Leydig cells. In each patient, concurrently with the advance in maturation of the scrotal testis, more pronounced changes took place in the undescended testis. The changes reported do not appear to be of genetic, endocrine or inflammatory origin, but can be traced directly to the abnormal position of the undescended testis.

SEVERAL investigators have studied the morphology of the undescended puberal testis (1–8) and have arrived generally at the conclusion that the outstanding anomalies consist of abnormally small tubules, underdevelopment of the germinal epithelium, sclerosis of the tubular wall and abnormal Leydig cells.

In a previous paper (9) we reported the histologic changes which take

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TABLE 1

Case No.	Age of boy	Position of undescended testis	Degree of testicular maturation
1	11 y. 8 m.	Upper portion of inguinal canal	First
2	10 y.	Over the internal inguinal ring	First
3	10 y.	Middle portion of inguinal canal	First
4	8 y.	Middle portion of inguinal canal	Second
5	12 y. 5 m.	Inferior portion of inguinal canal	Third
6	12 y. 10 m.	Over the internal inguinal ring	Third
7	12 y. 6 m.	Middle portion of inguinal canal	Third

place in normal puberal testes during the transitional period between prepuberal and postpuberal ages.

The present paper records a comparison of the histologic changes observed during puberal maturation of cryptorchid testes with those already described for normal puberal testes.

In our publications on the human testis, both normal and pathologic (9, 11–13), we have always stressed the importance of paying special attention to the connective tissue, in view of its fundamental role in testicular development and preservation. This same criterion is applied in the present study.

MATERIAL AND METHODS

Our investigation was carried out on 7 patients with true unilateral cryptorchism. Both the chronologic age of the patients and the position of the undescended testes are shown in Table 1. Biopsies were performed bilaterally on the same day.

With a view to enhancing the value of our data, the following conditions were borne in mind when selecting these cryptorchid patients: a) that they represent the puberal age, b) that their unilateral cryptorchism be due to mechanical obstruction, and c) that a control biopsy be carried out simultaneously on the normal scrotal testis.

The technics employed for staining histologic specimens were essentially the same as those described in our previous paper on puberal maturation of the normal human testis (9).

RESULTS[1]

The abnormalities found as a result of comparing the histologic findings in the undescended testis with those in the normal scrotal[2] testis of the

[1] The reader is referred to our previous paper entitled "Puberal Maturation of the Normal Human Testis" (this issue of the Journal (9)) before studying the results of present investigation. This recommendation is made for the following reasons: 1) the present findings are compared with the findings in normal puberal testes, and 2) the terms employed here to describe testicular maturation and the different types of cells are the same as those incorporated in the previous paper.

[2] It should be borne in mind that the scrotal testis in each of the patients exhibited the morphologic and histochemical characteristics previously reported for the normal puberal testis.

same subject can be divided into two fundamental groups: (a) changes which are merely suggestive of a delay in normal maturation, and (b) changes which reveal truly pathologic processes. For the sake of brevity, these changes will be referred to as: (a) delayed maturation, and (b) pathologic maturation.

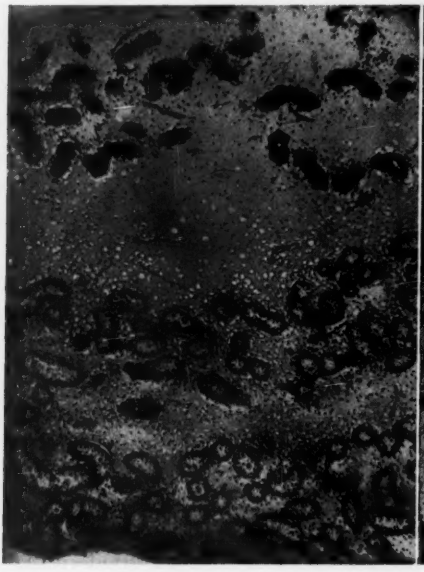

FIG. 1

FIG. 2

FIG. 1. (Case 1)—Scrotal testis. Normal testis showing first degree of maturation. Panoramic view of the three stages of development. In the upper part is a lobule in stage 1; in the center, a lobule in stage 2; in the lower part, a lobule in stage 3. (Hematoxylin-eosin ×69.)

FIG. 2. (Case 1)—Undescended testis. Tubules are of smaller diameter than in the scrotal testis, and the walls are slightly thickened in the lobule in stage 3. In all 3 stages the intertubular connective tissue appears more fibrillar. (Hematoxylin-eosin ×69.)

Histologic Findings

Case 1

Boy aged 11 years and 8 months. The scrotal testis[2] (Fig. 1) showed the first degree of maturation, with lobules in stages 1, 2 and 3 of development. The same three stages could also be seen in the undescended testis (Fig. 2).

Undescended testis—Tubules: In the stage-1 lobules, the tubules were of small diameter, the wall was slightly thickened, and there was an increase in the number of type "c" fibroblasts ("c.1" and "c.2"). Germinal epithelium was undifferentiated as yet in the scrotal testis. In stage-3 lobules, the tubular diameter was greatly reduced, and maturation of the germinal epithelium was delayed. Very few spermatogonia were found in the undescended testis, but they were abundant in the scrotal testis. The walls of

some tubules showed fibrillar and fibroblastic proliferation. Intertubular connective tissue: In the 3 lobular stages there was an increase in reticular fibers and type "c" fibroblasts and a simultaneous decrease in the number of type "b" fibroblasts (precursors of Leydig cells). No Leydig cells were discernible in either of the testes. There was partial sclerosis of the vessels corresponding to the albuginea.

Summary: Histologically, the undescended testis presented the following slight differences when compared with the scrotal testis: 1) *delay in maturation*—less differentiation of the germinal epithelium, tubules of smaller diameter, and fewer type "b" fibroblasts; 2) *pathologic maturation*—proliferation of fibrillae and type "c" fibroblasts in the tubular wall and intertubular connective tissue.

Case 2

Boy aged 10 years. The scrotal testis showed the first degree of maturation, with lobules in stages 1, 2 and 3 of development. The undescended testis also showed these three stages. The main difference was in the intertubular connective tissue.

Undescended testis—Tubules: When compared with the scrotal testis, no difference could be seen in stages 1 and 2 of lobular development, but the diameter of the tubules was somewhat smaller in the lobules in stage 3. Undifferentiated cells composed the tubular content, and some spermatogonia were seen in the scrotal testis. There was an increase in the number of reticular fibers in some of the tubular walls, but no increase in the quantity of fibroblasts. Intertubular connective tissue: There was a great increase in the number of "involutive" cells as compared with those in the scrotal testis (Figs. 3 and 4). These cells were often seen in clusters, chiefly in lobules in stage 1, although they were also present in lobules in stages 2 and 3. Stage-1 lobules also contained an abundance of transitional forms between type "a" fibroblasts and "involutive" cells. Stage-3 lobules, on the other hand, contained large numbers of transitional forms between type "b" fibroblasts and "involutive" cells. It should be remembered that, even in small quantities, these "involutive" cells are always found in the normal puberal testis. There was an increase in reticular fibers and type "c" fibroblasts, and a decrease in type "b" fibroblasts (precursors of Leydig cells). No Leydig cells were apparent either in the undescended or in the scrotal testis.

Summary: 1) *delay in maturation*—less differentiation of the germinal epithelium, tubules of smaller diameter, and a reduction in the number of type "b" fibroblasts; 2) *pathologic maturation*—numerous "involutive" cells, fibrosis, and proliferation of type "c" fibroblasts in the tubular wall and intertubular connective tissue.

Case 3

In this 10-year-old boy, both the scrotal and the undescended testis exhibited the first degree of maturation, with lobules in the three stages of development. However, there was a difference in their tubules as well as in the intertubular connective tissue of the stage-3 lobules.

Undescended testis—Tubules: A slight decrease in diameter was the only abnormality in the lobules in stages 1 and 2. In stage-3 lobules, there was a pronounced decrease in the tubular diameter. Their tubular content is made up of undifferentiated cells, whereas the scrotal testis contained abundant spermatogonia and even spermatocytes. There was thickening of the wall of some of the tubules. Intertubular connective tissue: No differences were observed in lobules in stage 1, but in stage-3 lobules there was fibrosis due to the increased number of reticular fibers. Moreover, an increase had taken place in type "c" fibroblasts, as against a decrease in type "b." Both testes contained a normal number of "involutive" cells, but no Leydig cells.

Summary: 1) *delay in maturation:* less differentiation of the germinal epithelium, decreased tubular diameter, and fewer type "b" fibroblasts; 2) *pathologic maturation:* proliferation of reticular fibers and type "c" fibroblasts.

Case 4

An 8-year-old boy. The morphology of the scrotal testis corresponded to that of lobular stage 3 of the first degree of maturation. On the other hand, in the undescended testis, all three stages of the first degree of maturation could be observed.

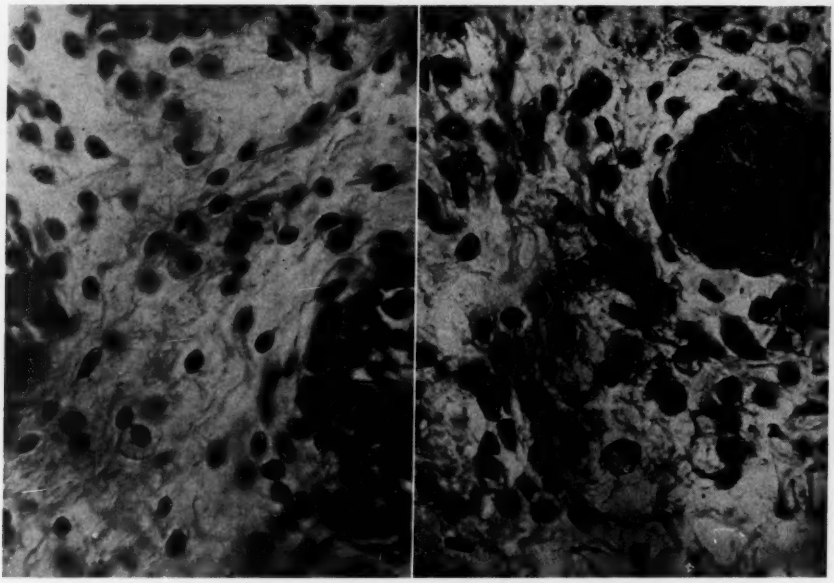

FIG. 3 FIG. 4

FIGS. 3 and 4. (Case 2)—Scrotal testis (Fig. 3) and undescended testis (Fig. 4). The histologic picture corresponds to that of stage-1 lobules of the normal testis in the first degree of maturation. There are numerous "involutive" cells and fibrosis of the intertubular connective tissue in the undescended testis. In the scrotal testis, few "involutive" cells can be seen, but type "b" fibroblasts are more numerous. (Hematoxylin-eosin ×645.)

Undescended testis—Tubules: These were markedly reduced in diameter. In the three stages of development the walls were very similar to those of normal tubules in the first degree of maturation. The tubular content in lobules in stages 1 and 2 consisted of undifferentiated cells only. Spermatogonia were present in lobules in stage 3, but even spermatocytes were found in the scrotal testis. Intertubular connective tissue: No differences were observed.

Summary: 1) *delay in maturation:* less differentiation of the germinal epithelium; 2) *pathologic maturation:* none.

FIG. 5

FIG. 6

FIG. 5. (Case 5)—The histologic picture in the scrotal testis corresponds in its entirety to that of a normal testis in the third degree of maturation. The tubules are of normal size, and the tunica propria and basal membrane are well developed. Germinal epithelium and Sertoli cells are present. Intertubular connective tissue cells have normal characteristics. (Hematoxylin-eosin ×69.)

FIG. 6. (Case 5)—Undescended testis. Lobules showing the three stages in a testis in the first degree of maturation. Stage 1 in the upper part, stage 2 in the middle, and stage 3 in the lower part. In stage 3 the tubules are of smaller diameter, and the wall is thicker as a result of fibroblastic proliferation in several layers. There is underdevelopment of the germinal epithelium. The intertubular connective tissue appears more fibrillar. (Hematoxylin-eosin ×69.)

Case 5

Boy aged 12 years and 5 months. The scrotal testis showed the third degree of maturation (Fig. 5). The undescended testis, however, was similar to the first degree of maturation, with lobules in the three stages of development (Fig. 6).

Undescended testis—Tubules: Their diameter was amaller. The walls, which were slightly thickened in the stage-1 lobules (Fig. 6), had become much thicker in stage 3, there being an increased amount of reticular fibers and juvenile fibroblasts arranged in several layers (Fig. 7). There was a delay in maturation in the germinal epithelium of the lobules in stage 3 (Fig. 6). Spermatogonia with pathologic features were seen occasionally (Fig. 7). Intertubular connective tissue: There were numerous cells with cytoplasmatic hypertrophy and vacuolization, their nuclei being normal or slightly abnormal (Fig. 8). These cells were found in clusters, and contained a large quantity

FIG. 7. (Case 5)—High-power microphotograph of area in Figure 6. Tubules have thickened walls consequent to fibrillar and fibroblastic proliferation. Tubular content shows delayed cellular maturation. Undifferentiated epithelial cells and some spermatogonia can be observed. (Hematoxylin-eosin ×300.)

of total lipids. "Small and compact" Leydig cells were present, but none of the "vacuolated" type, though the latter were found in the scrotal testis. There were more reticular fibers in all three stages of development (Fig. 6), and less type "b" but more type "c" fibroblasts. A few arterioles with thickened walls could also be seen (Fig. 7).

Summary: 1) *delay in maturation:* smaller tubular diameter, less development of the germinal epithelium, and delayed cellular maturation in the intertubular connective tissue; 2) *pathologic maturation:* pathologic spermatogonia, and sclerosis with proliferation of the fibroblasts in the tubular wall; in the intertubular connective tissue there were cells with cytoplasmatic hypertrophy and vacuolization, sclerosis, and proliferation of type "c" fibroblasts.

Case 6

Boy aged 12 years and 10 months. The scrotal testis exhibited the third degree of maturation (Fig. 9). Lobules of the undescended testis were similar to stage-3 lobules of testes in the first degree of maturation (Fig. 10).

Undescended testis—Tubules: Their diameter was somewhat reduced (Fig. 10).

FIG. 8. (Case 5)—High-power microphotograph of area in Figure 6. Abundant hypertrophied Leydig cells with cytoplasmatic vacuolization and some "small and compact" Leydig cells are discernible. (Hematoxylin-eosin ×300.)

Nearly all the tubules showed underdevelopment of the germinal epithelium and a predominance of spermatogonia with pronounced nuclear anomalies, whereas in the scrotal testis there were some spermatocytes, and even spermatids. There was a moderate thickening of the wall in nearly all the tubules (Fig. 10), and proliferation of type "c" fibroblasts. Intertubular connective tissue: There were abundant signs of cytoplasmatic hypertrophy and vacuolization (Fig. 10) when compared with the findings in the scrotal testis; most of the cells presented evidence of nuclear involution. "Involutive" cells were present in great quantities, and there were fewer normal Leydig cells. Type "c" fibroblasts were more numerous, but there was a smaller quantity of type "b" (Fig. 10). Thickened walls were observed in some of the arterioles.

Case 7

Boy aged 12 years and 6 months. The scrotal testis showed the third degree of maturation, whereas the undescended testis showed lobules in stage 3 of development in the first degree of maturation.

Undescended testis—Tubules: These were greatly reduced in diameter. They contained spermatogonia but many undifferentiated cells, whereas the scrotal testis contained even

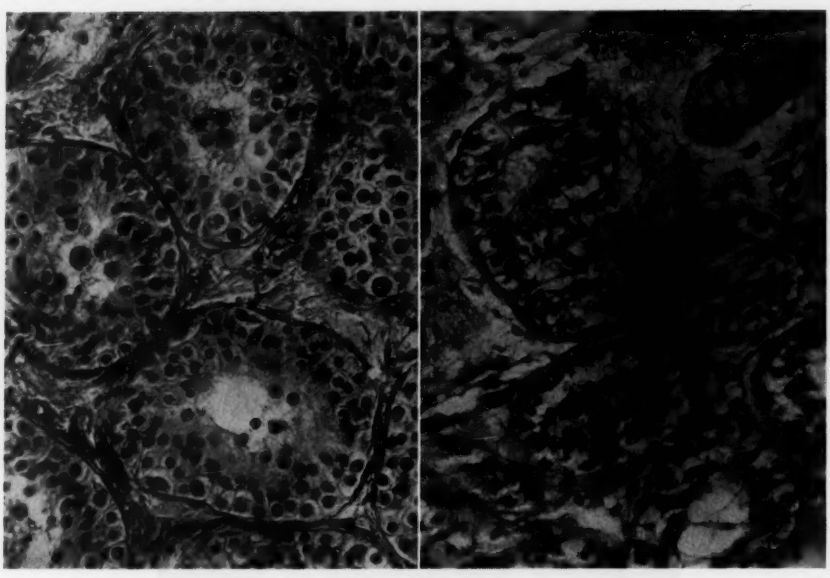

FIG. 9 FIG. 10

FIGS. 9 and 10. (Case 6)—The scrotal testis (Fig. 9) shows the third degree of maturation. The tubules of the undescended testis (Fig. 10) are of smaller diameter, and the germinal epithelium is not so well developed as it contains pathologic spermatogonia. There is slight thickening of the tubular wall. Large numbers of hypertrophied cells with cytoplasmatic vacuolization are seen. (Hematoxylin-eosin ×223.)

spermatocytes. The tubular wall was thickened due to an increased amount of reticular fibers and fibroblastic hyperplasia. Intertubular connective tissue: There were large numbers of cells with cytoplasmatic hypertrophy and vacuolization. "Involutive" cells, as well as "small and compact" Leydig cells, were also numerous. However, no vacuolated Leydig cells were observed, although they were evident in the scrotal testis.

Summary: 1) *delay in maturation:* less differentiation of the germinal epithelium, and no vacuolated Leydig cells; 2) *pathologic maturation:* an increased number of cells with cytoplasmatic hypertrophy and vacuolization in the intertubular connective tissue; peritubular and intertubular sclerosis with proliferation of type "c" fibroblasts.

COMMENTS

Our paper is limited to the histologic study of the undescended human testis during puberty, but it is of interest that both the clinical and hormonal findings were normal and coincided with those described by other investigators in the case of normal puberal youths (10).

Our study was prompted by a desire to establish the histologic differences between undescended and scrotal testes. The 7 cases were selected according to the following criteria: 1) that the patient be in the puberal age

group, 2) that the unilateral cryptorchism be due to mechanical obstruction, and 3) that (for control purposes) biopsies be carried out simultaneously on the scrotal and on the undescended testis in the same patient.

Only puberal cases were investigated, as it was considered essential to separate the study of undescended testes according to three important ages: infancy, puberty and adulthood. The puberal period was chosen, as in our experience the chronologic passage through normal puberty is accompanied by marked and rapid changes in the histologic pattern of the testes (9).

Our findings are confirmatory of those of other investigators, in the sense that testes not in the scrotum during puberty show abnormal histologic development, and that the changes noted when the patient is passing from the prepuberal age to adulthood differ from those described in normal puberal testes. These abnormalities appear in the fundamental structures of the organ, *i. e.*, tubular content, tubular wall and intertubular connective tissue, and are manifested by 1) delayed maturation, and 2) truly pathologic maturation.

Tubular content

Delay in normal maturation is associated with tubules of smaller diameter, and germinal epithelium with less numerous spermatogonia and decreased cellular maturation.

Pathologic maturation is attended by morphologic abnormalities in the cells of the germinal epithelium. Our studies have enabled us to confirm the reduced spermatic capacity reported by other authors. As in the case of other pathologic conditions of the testes studied by us (11–13), a very close relationship was observed between the intensity of the lesions in the tubular wall and those of the germinal epithelium—the thicker the tubular wall, the more intense the changes in the germinal epithelium.

Connective tissue of the tubular wall and intertubular spaces

Delay in normal maturation. In the intertubular connective tissue there was delay in normal maturation accompanied by arrest of cellular maturation in some of the stages previously described (9). For example, if there was an abundance of type "b" fibroblasts in the scrotal testis, there were very few in the undescended testis, in which type "c" fibroblasts predominated; and if there were "vacuolated" Leydig cells in the scrotal testis, there were only "small and compact" Leydig cells in the undescended one.

Pathologic maturation. Special significance is assigned to:

1) A simultaneous increase in the number of type "c" fibroblasts and collagen fibers in the intertubular connective tissue and in the tubular wall. This phenomenon would appear to be the expression, in the testis, of a gen-

eral process of maturation of the connective tissue in other parts of the body under normal and pathologic conditions, *i.e.*, the transition from immature fibroblasts to adult ones is accompanied by fibrilogenesis.

2) An increase in the number of "involutive" cells (which appear to originate from type "b" fibroblasts and from the "small and compact" Leydig cells) and also in the number of cells with cytoplasmatic hypertrophy and vacuolization. We lean towards the opinion that the increases in these two types of cells have a common pathogenesis, being the expression of an arrest in the maturation of the interstitial cells, accompanied by fatty degeneration. It should be remembered that, though in small numbers, these types of cells exist in the normal puberal testis. Summing up, in the undescended testis we observed alterations in the cellular and fibrillar components of the intertubular connective tissue. From the point of view of androgenic activity, the testicular cells showed morphologic and histochemical changes; but neither the urinary excretion of neutral 17-ketosteroids nor the clinical signs in our patients indicated androgenic insufficiency. This may have been due to the fact that we were dealing with *unilateral* cryptorchism.

3) A greater thickness of the tubular wall owing to an increase in the number of reticular and collagen fibers, and type "c" fibroblasts.

Pathologic changes were observed in the three fundamental sections of the testes. The degree of delayed maturation and of pathologic maturation varied in each case. A close relationship was found between delayed maturation and pathologic maturation in the cells of the intertubular connective tissue, as manifested by a decrease in the number of type "b" fibroblasts and a greater quantity of type "c" fibroblasts.

It is interesting that the nearer the scrotal testis came to reaching maturity, the greater the difference between it and the undescended testis. When the scrotal testis showed the first degree of maturation, the undescended one showed a delay in maturation but only slight manifestations of pathologic maturation (Figs. 1 and 2). On the other hand, when the scrotal testis showed the third degree of maturation, the pathologic maturation of the undescended testis became more evident (Figs. 5 and 6).

The pathogenesis of the changes found in the undescended testes may possibly be related to the temperature resulting from their abnormal localization. Moore (14) was the first to demonstrate the unfavorable influence of high temperatures on the germinal epithelium. In support of this theory is the fact that in all our cases surgical operation revealed mechanical obstructions which prevented spontaneous testicular descent. The fact that the scrotal testes were histologically normal and that the cryptorchism was unilateral in each patient would be against the theory of a dysgenic origin of the anomaly. However, we agree with Sohval's opinion (2) that "dys-

genic testicular lesions are of etiologic significance in some cases of cryptorchism."

The patients studied were not suffering from any endocrine disease nor was there anything significant in their general health which could account for the changes found in their testes. The histologic lesions were not of an inflammatory nature, nor were they of the type seen in Klinefelter's syndrome or in testicular aplasia.

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FUNCTIONING METASTATIC BRONCHIAL CARCINOID WITH ELEVATED LEVELS OF SERUM AND CEREBROSPINAL FLUID SEROTONIN AND PITUITARY ADENOMA

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ABSTRACT

A patient is described who had metastasizing bronchial carcinoid, hyperserotonemia, and an elevated urinary excretion of 5-hydroxy-3-indole acetic acid in the absence of the clinical characteristics of the carcinoid syndrome. During administration of reserpine there was an initial 5-hydroxy-3-indoluria, and excretion remained at levels above normal. A carcinoid metastasis to the choroid of the eye was found at operation, and there was possible metastatic involvement of the brain, based on an elevated level of serotonin in the cerebrospinal fluid. Associated was a pituitary adenoma with clinical evidence of acromegaly. It is suggested that patients with bronchial carcinoid undergo serotonin studies whether the carcinoid syndrome be present or not, and that these cases be scrutinized for additional endocrine adenomas.

THE carcinoid syndrome associated with metastasizing bronchial adenoma was recently described by Warner and Southren (1). The present report deals with an additional patient, in whom hyperserotonemia and an increase in the urinary excretion of 5-hydroxy-3-indole acetic acid was found in the absence of the clinical characteristics of the carcinoid syndrome. There was also an elevation of the serotonin content of the cerebrospinal fluid, a finding which suggested metastatic involvement of the brain. A pituitary adenoma with clinical evidence of acromegaly added to the unusual features of this case.

CASE REPORT

L.S., a 22-year-old white woman was first seen in the Endocrine Clinic of The Mount Sinai Hospital in July 1957 complaining of a 50-pound weight gain which had occurred during the preceding year. In April 1950, fever and chills had developed. Physical examination at that time was reported to have revealed the presence of rales over the left hemithorax. A diagnosis of pneumonia was made and the patient was treated by antibiotics, with initial improvement. However, during the next several months, recurrent

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febrile episodes were experienced. A roentgenogram of the chest showed a shadow in the left upper lung field and she was admitted to The Mount Sinai Hospital in September 1950 for evaluation.

Bronchoscopy revealed the presence of a mass obstructing the left upper-lobe bronchus. Microscopic examination of the biopsy material obtained from the mass indicated a carcinoid type of bronchial adenoma (Figs. 1 and 2). Physical examination disclosed dullness to percussion and decreased breath sounds over the left upper lung field anteriorly. There were no flushes, diarrhea, hypertension or cardiomegaly. In October 1950, a *left pneumonectomy* was performed. Microscopic examination of the resected specimen confirmed the biopsy finding of bronchial carcinoid. The wall of the bronchus

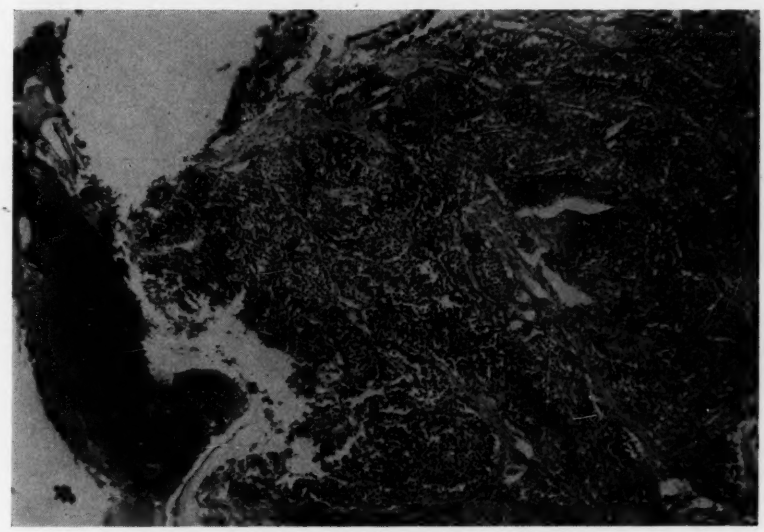

FIG. 1. Fragment of bronchial adenoma obtained from biopsy specimen. (Hematoxylin-Eosin ×58.)

was infiltrated with tumor cells. The patient's postoperative course was uneventful.

In 1954 oligomenorrhea developed, with intervals between periods varying from two or three to as long as nine months. Initially, withdrawal bleeding occurred after intramuscular injections of 17-hydroxyprogesterone caproate. However, by 1956, complete amenorrhea had developed and estrogen as well as progesterone was necessary to induce withdrawal bleeding. Between 1956 and 1957 there was a weight gain of 50 pounds, associated with hirsutism between the breasts and gradual enlargement of the hands and feet. On one occasion, during an examination, a small amount of whitish fluid could be expressed from the breasts. In March 1957 she complained of blurring vision in the right eye. Funduscopic examination revealed a detachment of the retina in the temporal field. Intravenous injection of P^{32} (2) with differential counting over the affected eye (using the normal eye as a control) indicated tumor tissue. The patient was again admitted to The Mount Sinai Hospital on May 8, 1957, for surgical exploration of the eye. Roentgenograms of the chest showed postpneumonectomy changes, with no apparent

mediastinal shift. The right lung appeared to be normal. Skeletal survey disclosed no bony abnormality. Examination of the skull, however, showed the sella turcica to be distinctly enlarged with thinning of the posterior clinoid processes.

Laboratory studies disclosed normal values for the blood hemoglobin level, white blood cell count and differential, blood serologic tests, urinalysis, levels of blood urea nitrogen, fasting blood sugar, total serum cholesterol, serum electrolytes, calcium, phosphorus, alkaline phosphatase, bilirubin, and cephalin flocculation. The thyroidal uptake of I^{131} after twenty-four hours was 28 per cent and the plasma protein-bound iodine level

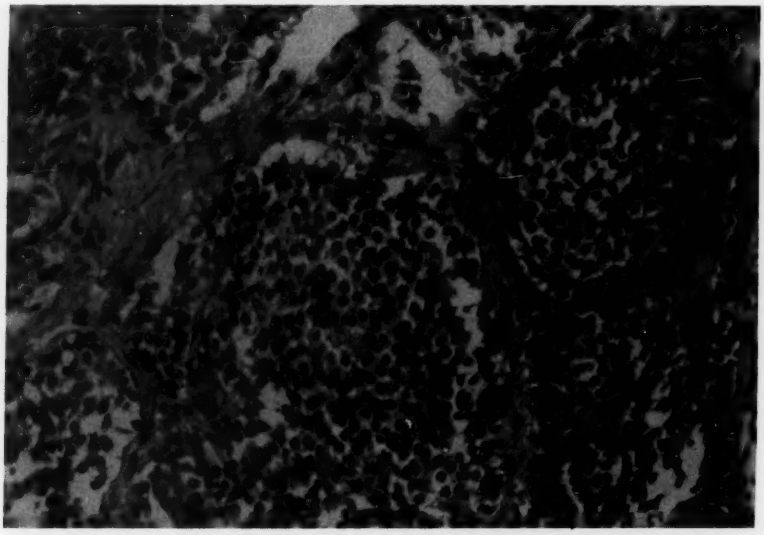

FIG. 2. High-power view of the tumor showing the uniformity of cellular structure and the round vesicular nuclei with little hyperchromatism and rare mitotic figures, typical of bronchial carcinoid. (Hematoxylin-Eosin, ×196.)

was 4.5 μg. per 100 ml. The 24-hour urinary excretions of 17-hydroxycorticoids and neutral 17-ketosteroids were 10.3 and 12.9 mg. respectively. Urinary gonadotropin titers were positive at 5 and 10 mouse uterine units and negative at 20, 50 and 100 mouse uterine units. Results of a water load test were within normal limits (3).

On May 17, 1957, an *enucleation of the right eye* was performed. In the globe was a nonpigmented choroidal mass in the upper temporal area underlying the previously described retinal detachment (Fig. 3). Microscopic examination (Fig. 4) revealed the tumor tissue to be a carcinoid type of bronchial adenoma identical with the bronchial tumor removed eight years previously. The postoperative course was uneventful. In June 1957, a course of radiotherapy was administered to the pituitary, consisting of 4000 roentgens.

Upon physical examination in the Endocrine Clinic in July 1957 the patient was a tall obese white woman with coarsened facial features, spade-like hands, and large feet. Her height was 175 cm., weight 196 pounds, oral temperature 99.0° F., radial pulse regular at 80 beats per minute, and blood pressure 125/90 mm. Hg. There was an ocular pros-

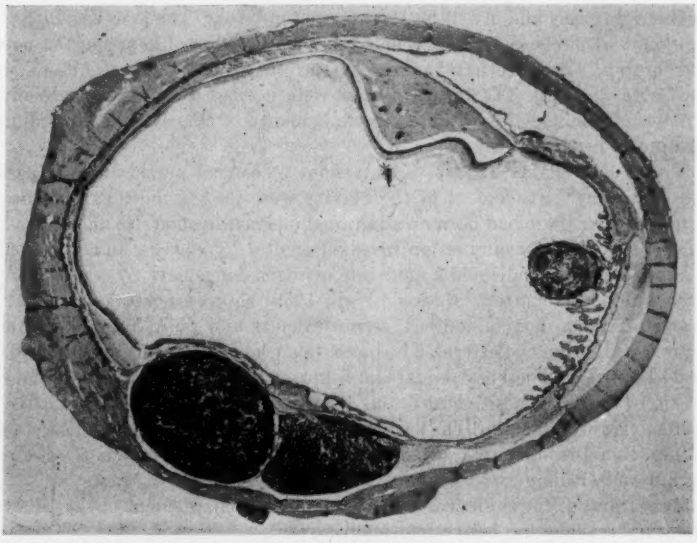

FIG. 3. A section through the globe of the eye showing 2 well demarcated subretinal tumor masses. (Courtesy of the Ophthalmology Dept., The Mount Sinai Hospital, New York City.)

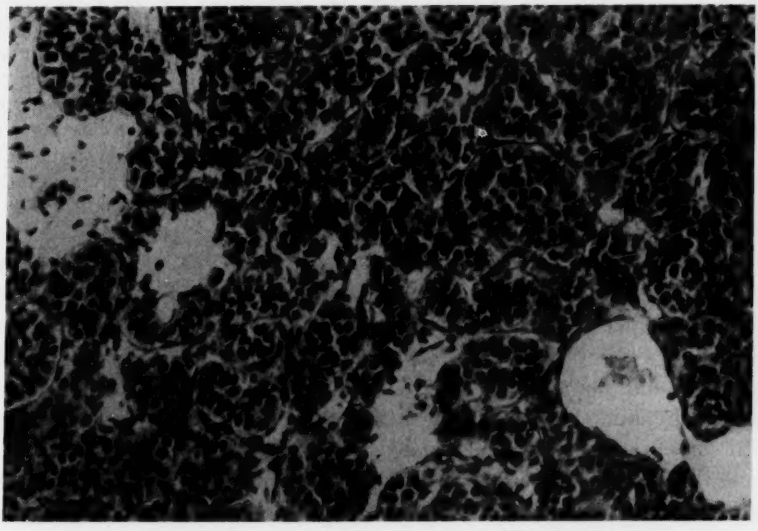

FIG. 4. Section of the ocular tumor demonstrating the striking similarity of its cellular characteristics to those of bronchial carcinoid. (Hematoxylin-Eosin, ×196. Courtesy of the Ophthalmology Dept., The Mount Sinai Hospital, New York City.)

thesis in the right orbit and a well healed thoracotomy scar. The skin was moist, and a normal amount of pubic and axillary hair was present. Some hirsutism was noted between her breasts. Over the left hemithorax the breath sounds were absent and the percussion note was dull. The breath sounds were normal over the right hemithorax. The heart was not enlarged and there was no evidence of congestive heart failure, cardiac murmurs or hepatomegaly.

From July 1957 to May 1959 no spontaneous menstrual periods were experienced. Attempts at dietary management of the obesity were for the most part unsuccessful. During the last year there had been an additional increase in shoe size and in the hoarseness of her voice. Pelvic examination revealed genital hypoplasia and a normal sized clitoris. A vaginal smear indicated a moderate estrogen deficiency.

The most recent laboratory studies (April, 1959) showed normal values for blood urea nitrogen, carbon dioxide content, serum calcium and phosphorus, alkaline phosphatase and total serum cholesterol. The blood sugar level was 92 mg. per 100 ml. at the end of a 24-hour fast. The basal metabolic rate was 0 per cent, and the urinary gonadotropin titers were still positive at 5 and 10 mouse uterine units and negative at 20, 50 and 100 mouse uterine units. The 24-hour urinary excretion of neutral 17-ketosteroids was 12.6 mg. Renal tubular reabsorption of phosphorus was 98 per cent. An electroencephalogram was within normal limits.

Roentgenograms of the skull again showed moderate enlargement of the sella turcica. Skeletal survey revealed no bony abnormalities.

Metabolic Studies

The patient was maintained on a banana-free diet. The concentrations of serotonin in the cerebrospinal fluid and blood serum were determined with the use of the isolated uterus of an estrogenized rat as a test object (4). Identity of the biologically active material as serotonin was demonstrated by its inhibition with 1-benzyl-3,5-dimethyl serotonin, a potent antiserotonin. Determination of the 5-hydroxy-3-indole acetic acid content of the urine was carried out by the colorimetric method of Udenfriend *et al.* (5). Following collection of a control sample of serum and a 24-hour urine specimen, reserpine was administered to the patient intramuscularly in a dose of 1.0 mg. once daily for seven days. During this period, 24-hour urine specimens were collected. Corresponding urines were also obtained from a normal subject before and during reserpine administration.

RESULTS

The initial level of serotonin in the blood serum of Patient L. S. (Table 1) was 1.20 μg. per ml. (normal, 0.10 to 0.40 μg. per ml.), and this fell to less than 0.030 μg. per ml. by the fifth day of reserpine therapy. The urinary excretion of 5-hydroxy-3-indole acetic acid (Table 1) increased from an initial level of 21.3 mg. per twenty-four hours (normal, 1 to 9 mg.) to 26.2 mg. on the third day, and then decreased to 17.6 mg. per twenty-four hours by the fourth day of reserpine administration. Continued administration of reserpine failed to produce any further decrease in the urinary excretion of 5-hydroxy-3-indole acetic acid. The value on the seventh day was well above the normal range. The control subject showed a similar decline in the serum level of serotonin, and the excretion of 5-hydroxy-3-indole acetic acid showed a small diminution preceded by a slight initial rise. There were

TABLE 1. EFFECT OF RESERPINE ON SEROTONIN LEVEL OF SERUM AND CEREBROSPINAL FLUID, AND EXCRETION OF 5-HYDROXY-INDOLE ACETIC ACID IN PATIENT L.S.; CONTROL STUDIES IN PATIENT N.P.

Subject	Day of study	Serotonin in blood serum* (μg./ml.)	Urinary 5-hydroxy-3-indole acetic acid† (mg./24 hrs.)	Serotonin in cerebrospinal fluid‡ (μg./ml.)	Medication
L.S. (bronchial carcinoid)	0	1.20	21.3	0.035	No medication (control)
	1		23.8		Reserpine, 1 mg./d intramuscularly
	2		25.7		
	3		26.2		
	4		17.6		
	5	<0.03	17.8		
	6		17.5		
	7		17.4		
N.P. (normal)	0	0.40	3.5		No medication (control)
	1		4.2		Reserpine, 1 mg./d intramuscularly
	2		3.2		
	3		2.5		
	4		2.4		
	5	<0.03	2.7		

* Normal, 0.10–0.40 μg./ml.
† Normal, 1–9 mg./24 hrs.
‡ Normal, <0.01 μg./ml.

no clinical side-effects attributable to the drug. The concentration of serotonin in the cerebrospinal fluid of Patient L.S. was 0.035 μg. per ml. (normal, less than 0.01 μg. per ml. (6, 7)), and the protein content was 28 mg. per 100 ml.

COMMENT

Shore (8), Pletscher (9) and their associates showed that the administration of reserpine to animals caused a release of serotonin from the body depots. The urinary excretion of the serotonin metabolite, 5-hydroxy-3-indole acetic acid, increased initially and then decreased to below normal levels. Haverbach *et al.* (10) demonstrated that part of this depletion was related to a marked reduction in platelet serotonin.

The present study has revealed the ready depletion of serotonin in the blood serum following reserpine therapy, in both the carcinoid patient and in the normal subject. There was a slight reduction in the urinary excretion of 5-hydroxy-3-indole acetic acid in the normal subject. In the carcinoid patient, however, urinary excretion of 5-hydroxy-3-indole acetic acid remained moderately elevated despite more prolonged reserpine administration. Other investigators have obtained similar results (11, 12). These data suggest that the serotonin-releasing property of reserpine is at most an initial transient effect limited to the platelets and other labile body stores.

The major body store (in this instance, the tumor) is apparently more resistant to depletion.

An interesting and somewhat unexpected finding was the elevated level of cerebrospinal fluid serotonin. Previously reported cases of metastatic carcinoid did not show elevated cerebrospinal levels (11) despite the high blood content. Intravenous infusion of large amounts of serotonin in a human subject (7) likewise failed to raise the cerebrospinal fluid level. A number of subjects with a variety of primary brain tumors (7, 13), however, did show moderate to marked elevations of cerebrospinal fluid serotonin, although in several patients with isolated pituitary adenomas there was no detectable increase (7). The findings in our patient would suggest the presence either of retro-orbital functioning carcinoid tissue or brain metastasis. Among the known metastatic sites of bronchial carcinoid (regional lymph nodes, liver, bone marrow and other organs), involvement of the brain and the choroid of the eye have not been previously reported.

The association of bronchial and pituitary tumors has been noted as an incidental finding in 2 previous cases of multiple endocrine adenomas (14, 15). However, no serotonin studies were performed. It is suggested that patients with bronchial carcinoid undergo serotonin studies, whether the carcinoid syndrome be present or not, and that these cases be scrutinized for additional endocrine adenomas.

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URINARY GONADOTROPIN EXCRETION MEASURED BY THE MOUSE UTERINE RESPONSE, EMPLOYING ESTRONE AS THE STANDARD REFERENCE MATERIAL*†

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ABSTRACT

Human urinary pituitary gonadotropin (HPG) levels in normal male and female subjects were measured by the mouse uterine response, employing estrone as the standard reference material. The range of error for each determination when 40 mice were used was ±25 per cent at P: 0.95. Single tests performed on 27 male subjects, 14 to 43 years of age, demonstrated that HPG excretion levels, when positive, ranged from 0.2 to 2.4 μg. of estrone equivalent per twenty-four hours (a 12-fold span). In 11 per cent of the subjects the values were negative. Data on consecutive urine samples from normal men indicated that the daily excretion of HPG was not constant. Single tests performed on 11 normal menstruating women, 14 to 42 years of age, demonstrated that, when positive, HPG levels ranged from 0.6 to 2.3 μg. of estrone equivalent per twenty-four hours (a 3.8-fold span). In 36 per cent of these subjects the values were negative. The pattern of HPG excretion during one menstrual cycle was not constant in the 8 women studied. Thirty-seven per cent of the subjects showed a significant peak on the fourteenth to sixteenth days of the cycle. Negative values were observed in 10-year-old subjects (2 males and 1 female). When daily consecutive samples of urine from postmenopausal women were studied, a cyclic excretion of HPG was observed; the magnitude of the daily variation ranged from 3.4 to 12.7 fold. No diurnal rhythm was noted.

THE necessity of using a standard for meaningful interpretation of values obtained for human urinary pituitary gonadotropin (HPG) in studies of human gonadotropic function has been repeatedly emphasized (1–4).

The absence of an international standard, together with the common use of a small number of animals without precise statistical evaluation, has fre-

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quently led to rough estimates of the titer of gonadotropin in human urine rather than to accurate meaningful quantitative determinations.

We adopted estrone as a convenient, reproducible, easily available reference standard (5). The method, which employs the mouse uterine response, provides a sensitive and accurate bioassay for the estimation of total HPG excretion. Using this method, the HPG excretion levels of normal men, normal menstruating women and normal postmenopausal women were studied. Results are expressed as μg. of estrone equivalent per twenty-four hours.

MATERIAL AND METHODS

Urine from normal men (14 to 43 years of age) and from normal menstruating women and normal postmenopausal women (14 to 72 years of age) was collected in 24-hour specimens, using toluene as preservative. Urine from male and female subjects before the age of puberty was also collected. Specimens were frozen at −14° until processed.

Due to the low excretion of HPG in normal men and in normal menstruating women, it was necessary to work with pools of three-day urine collections for the quantitative estimation of urinary HPG in these subjects. Single 24-hour urine collections were used for quantitative estimations in postmenopausal women.

The kaolin adsorption method described by Albert (6) was used. A slight modification was introduced by replacing the automatic self-regulating filter apparatus with a Buchner funnel attached to a filter flash which was coupled to a suction pump.

The final quantitative estimations were carried out according to a method previously described (5). For each assay three doses of the standard (estrone) and two doses of the urinary concentrates (3 and 2 design) were used, each dose being given to a group of 11 mice.

The doses of the standard were: 0.05 μg., 0.1 μg. and 0.2 μg. per animal. The doses of the concentrates were: 2-hour and 4-hour equivalents of urine per animal by weight of the 72-hour precipitate when determinations were made on the urine of men and of normal menstruating women, and 0.25-hour and 0.5-hour equivalents of urine per animal by weight of the 24-hour precipitate when determinations were made on the urine of postmenopausal women.

Statistical evaluation of the data was carried out according to the methods of Fisher (7), Bülbring (8) and Emmens (9).

RESULTS

Figure 1 shows single HPG assay values plotted as a function of age, for each of 27 normal males. Negative responses were observed in two 10-year-old subjects and one 25-year-old subject. Positive values ranged from 0.2 to 2.4 μg. of estrone equivalent per twenty-four hours, a 12-fold span. The mean of the individual deviations in relative potency as obtained by the confidence limits of P:0.95 was 22.3 per cent irrespective of the sign.

Figure 2 shows the excretion of HPG in a 38-year-old man followed intermittently for several months. The pattern of excretion was not constant. Negative values were observed on five occasions. Otherwise, the values ranged from 0.36 to 3.1 μg. of estrone equivalent per twenty-four hours, an

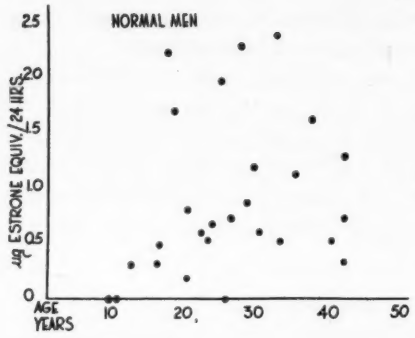

FIG. 1. Single assay values of HPG plotted as a function of age, for each of 27 normal male subjects.

8.6-fold span. During this study a significant variation in HPG excretion was observed four times when consecutive samples were studied. The mean of the individual deviations in relative potency as obtained by the confidence limits of P:0.95 was 24.5 per cent irrespective of the sign.

Figure 3 shows the excretion of HPG in a 22-year-old man followed almost consecutively for three and a half months. Negative values were observed twice. Positive values ranged from 0.2 to 2.14 μg. of estrone equivalent per twenty-four hours, a 10-fold span. A significant variation in HPG excretion was observed when 8 consecutive samples were studied. On one occasion the precipitate was toxic, and on another occasion one sample elicited the maximum response at the dose levels tested. The mean of the individual deviations in relative potency as obtained by the confidence limits of P:0.95 was 19.2 per cent irrespective of the sign.

Figure 4 shows the excretion of HPG in a 21-year-old man followed intermittently for four months. Negative titers were observed four times. Positive values ranged from 0.3 to 0.76 μg. of estrone equivalent per twenty-four hours, a 2.5-fold span. A significant variation in HPG excretion was observed twice when consecutive samples were studied. The mean of the

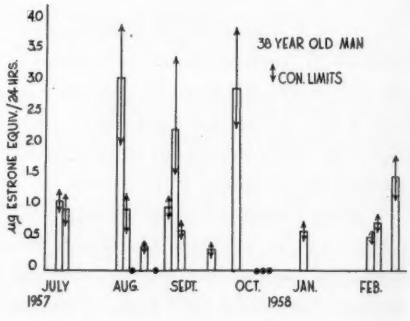

FIG. 2. HPG excretion values in a 38-year-old man followed for six months.

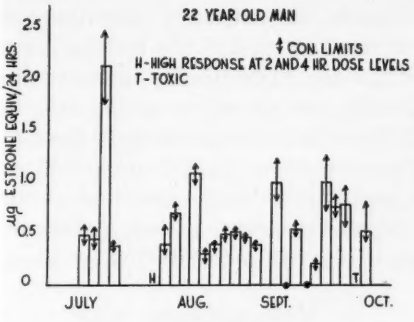

FIG. 3. HPG excretion values in a 22-year-old man followed for four months.

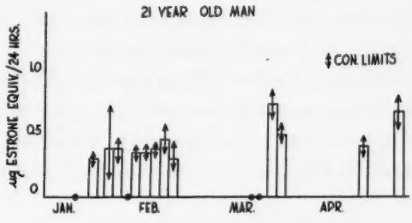

FIG. 4. HPG excretion values in a 21-year-old man followed for four months.

individual deviations in relative potency as obtained by the confidence limits of P:0.95 was 24.5 per cent irrespective of the sign.

Figure 5 shows single assay values for HPG excretion obtained on each of 16 normal female subjects 10 to 72 years of age. The group with regular menstrual cycles collected urine specimens on the first to the third and on the fourteenth to the sixteenth day of the cycle. The titers were negative in a 10-year-old girl as well as in 3 subjects 14, 18 and 23 years old, respectively, when studied on the first to the third day of the cycle, and in an 18-year-old subject studied on the fourteenth to the sixteenth day of the menstrual cycle. Positive values ranged from 0.6 to 2.3 μg. of estrone equivalent per twenty-four hours, a 3.8-fold span. Values obtained in postmenopausal women, 50 to 72 years of age, ranged from 2.8 to 23.0 μg. of estrone equivalent per twenty-four hours, an 8.2-fold span. The mean of the individual deviations in relative potency as obtained by the confidence limits of P:0.95 was 19.4 per cent irrespective of the sign.

Figures 6 and 7 show HPG values obtained on 8 normal female subjects,

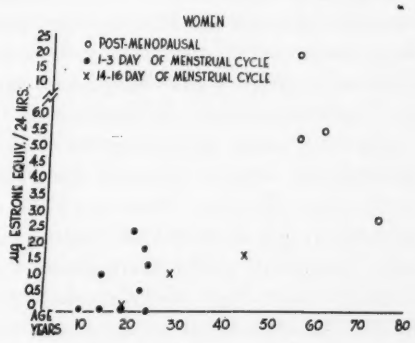

FIG. 5. Single assay values of HPG for each of 16 normal women.

16 to 42 years old, during one menstrual cycle. The pattern of excretion was not constant. From Figure 6 it may be seen that in 3 of the women there was a significant peak on the fourteenth to the sixteenth day of the cycle. In the fourth subject the level of excretion was the same on the first to third, fourteenth to sixteenth, and twenty-fifth to twenty-seventh days of the cycle. In the fifth subject, HPG was excreted on the first to third days, but not on the fourteenth to sixteenth, and twenty-fifth to twenty-seventh days of the cycle. In the sixth subject the values were negative. As may be seen in Figure 7, the seventh subject showed a high gonadotropin excretion

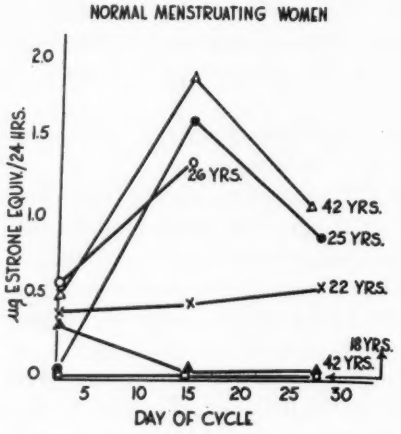

FIG. 6. HPG excretion in 6 normally menstruating women during one menstrual cycle.

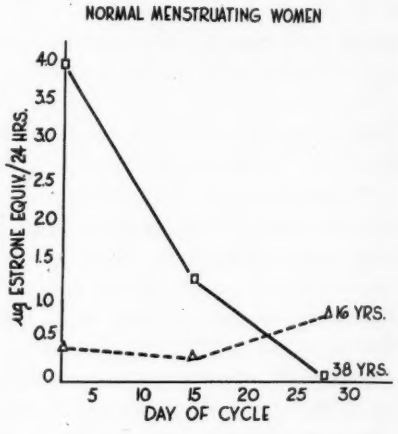

FIG. 7. HPG excretion values in 2 normally menstruating women during one menstrual cycle.

on the first to third days of the cycle, with a significant decrease on the fourteenth to sixteenth days and a negative titer on the twenty-fifth to twenty-seventh days of the cycle. In the eighth subject the level of HPG excretion was the same on the first to third days as on the fourteenth to sixteenth days, but rose on the twenty-fifth to twenty-seventh days of the cycle. The mean of the individual deviations in relative potency as obtained by the confidence limits of P:0.95 was 16.5 per cent irrespective of the sign.

Figure 8 shows the excretion of HPG in Subject A, a 56-year-old postmenopausal woman followed daily for twenty-seven days. Negative titers were observed twice. Positive values ranged from 1.97 to 25 μg. of estrone equivalent per twenty-four hours. There was a cyclic variation in excretion: three-day cycles were present five times; four-day cycles, twice; a five-day cycle, once; and a six-day cycle, once. The magnitude of the daily fluctuation measured as the span between the lowest and highest values

was 12.7-fold. Partial overlap in confidence limits occurred in 5 of 27 assays. The mean of the individual deviations in relative potency as obtained by the confidence limits of P:0.95 was 19.3 per cent irrespective of the sign.

Figure 9 shows the pattern of HPG excretion in Subject B, a 59-year-old postmenopausal woman followed daily for fifteen days. There were no negative titers. Positive values ranged from 3.5 to 11.9 μg. of estrone equivalent per twenty-four hours. There was a cyclic variation in excretion; a three-day cycle was present once; a four-day cycle, twice; and a six-day cycle, once. The magnitude of the daily variation was 3.4-fold. There was

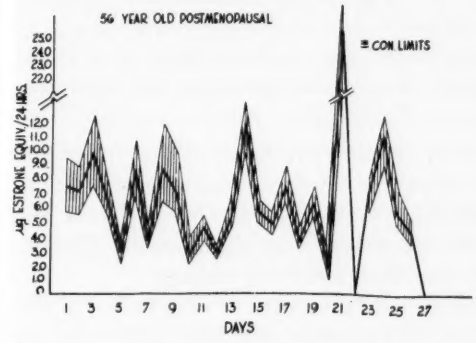

FIG. 8. Daily consecutive HPG assays in a 56-year-old postmenopausal woman (Subject A).

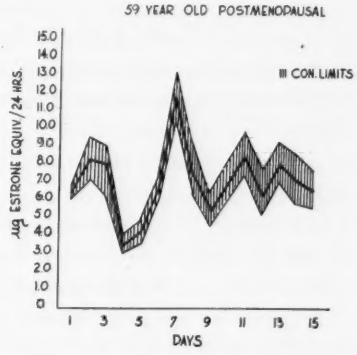

FIG. 9. Daily consecutive HPG assays in a 50-year-old postmenopausal women (Subject B).

partial overlap in confidence limits in 8 of 15 assays. The mean of the individual deviations in relative potency as obtained by the confidence limits of P:0.95 was 14.7 per cent irrespective of the sign.

Figure 10 shows the pattern of excretion in Subject C, a 50-year-old postmenopausal woman followed daily for twenty-one days. Negative titers were observed twice. Positive values ranged from 1.18 to 7.72 μg. of estrone equivalent per twenty-four hours. A cyclic variation in HPG excretion was observed; a four-day and an eleven-day cycle were each present once. There was overlap in confidence limits in 7 of 21 assays. The magnitude of the daily variation was 6.5-fold. The mean of the individual deviations in relative potency as obtained by the confidence limits of P:0.95 was 13.5 per cent irrespective of the sign.

Figure 11 shows the pattern of HPG excretion in Subject D, a 52-year-old postmenopausal woman followed daily for nine days. Negative titers were observed only once. HPG values ranged from 2.67 to 11.94 μg. of estrone equivalent per twenty-four hours. During the period of study a

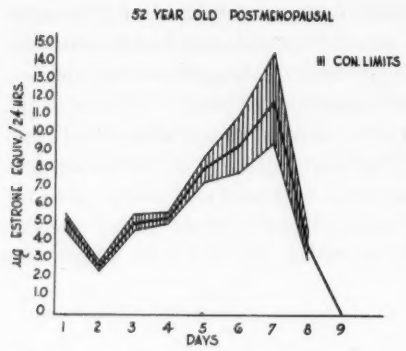

FIG. 10. Daily consecutive HPG assays in a 50-year-old postmenopausal woman (Subject C).

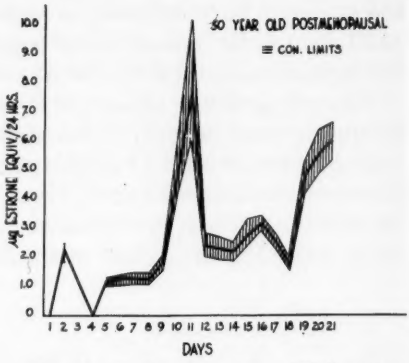

FIG. 11. Daily consecutive HPG assays in a 52-year-old postmenopausal woman (Subject D).

gradual increase in HPG excretion occurred from day 2 to day 7, but the titers became negative on day 9. There was overlap in confidence limits in 4 of 9 assays. The magnitude of the daily variation was 4.5-fold. The mean of the individual deviations in relative potency as obtained by the confidence limits of P:0.95 was 13 per cent irrespective of the sign.

Subject A collected 12-hour specimens of urine from 8 A.M. to 8 P.M. and from 8 P.M. to 8 A.M. for eight days, on two occasions. The HPG data are presented in Figure 12. HPG excretion values were similar in day and night specimens on five occasions; the values in day specimens were higher than those in night specimens six times; and the values in night specimens were higher than those in day specimens five times. The average HPG value for the day specimens was 5.72 μg. of estrone equivalent per twenty-four hours and the average value for the night specimens was 6.1 μg..

COMMENTS

The variation in HPG excretion in nonpregnant subjects as determined

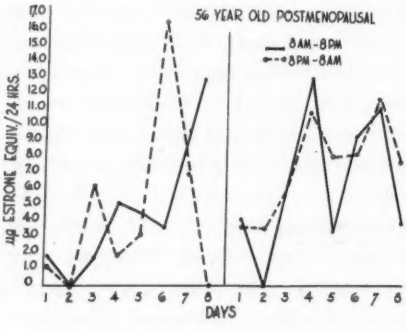

FIG. 12. HPG values in consecutive night-and-day samples of urine from a 56-year-old postmenopausal woman (Subject A).

in this study is in agreement with results reported in the literature (2–10).

Single observations of HPG excretion in men, demonstrated a 12-fold variation; the incidence of negative titers was 11 per cent. The results were similar when male subjects were followed over a prolonged period of time. In single estimations of HPG excretion in menstruating women there was a 3.8-fold variation; the incidence of negative titers was 36 per cent. In single observations in postmenopausal women, the variation was 8-fold. Most of the values obtained in postmenopausal women were higher than the upper limit for normal men and normal menstruating women, but here again, negative titers occurred when these subjects were studied over a prolonged period of time.

With respect to HPG excretion during the menstrual cycle, our observations did not demonstrate high levels in midcycle in the 8 subjects studied. The data do not correlate the ovulatory period and/or respective lengths of the follicular and luteal phases of the cycle with a "peak" of HPG excretion, inasmuch as the assessment of the time of ovulation was not attempted.

The scheduled time for urine collections was arbitrarily chosen. The criterion used was the regularity and length of previous menstrual cycles. Only 37 per cent of the female subjects exhibited a significant peak on the fourteenth to sixteenth days of the cycle.

The use of three-day urinary pools for estimation of HPG brings about a dilution factor which distorts any attempt to estimate accurately a possible "peak" of HPG excretion.

The occurrence of a cyclic variation in HPG excretion in postmenopausal women is in accordance with the findings of Albert *et al.* (10, 11). When postmenopausal women were studied for fifteen to twenty-seven consecutive days, the HPG cycles varied from three to eleven days in length. This pattern was not observed in a subject followed for nine days. The presence of a diurnal rhythm was not indicated when fractional urine specimens from a postmenopausal woman were investigated.

Previous work (2, 3, 4–12) has shown that it is possible to use a gonadotropin extract of urine from men and postmenopausal women as a standard preparation for similarly prepared gonadotropin extracts from nonpregnant subjects. Such preparations are not available at present to conduct long-range studies. For this reason we adopted estrone as a convenient and easily available standard. This study provides a precise evaluation of HPG excretion, and demonstrates the importance of the use of a standard for meaningful interpretation of assays in studies of human pituitary function. The range of error for each determination when 40 mice were used was ± 25 per cent at P:0.95.

Acknowledgments

Acknowledgment of devoted technical assistance is made to Mrs. Anna Zisch, Mr. Izo Engel, Mr. John T. Larson and Mr. Richard Leard.

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THE COMBINED ESTIMATION OF CORTISOL AND 11-DESOXYCORTISOL IN PLASMA AS PORTER-SILBER CHROMOGENS

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ABSTRACT

Current methods of measuring the concentration of plasma 17-hydroxycorticosteroids, although adequate for cortisol, may vary in the recovery of 11-desoxycortisol. The present study demonstrates that procedures which employ solvent partition to transfer 11-desoxycortisol from the extracting solvent, dichloromethane, into the phenylhydrazine-sulfuric acid-ethanol color reagent lose almost 50 per cent of the steroid in this step. A modification of the Silber-Porter procedure has been developed, which overcomes this difficulty by evaporating the extracting solvent before addition of the color reagent. By the joint quantitation of both cortisol and 11-desoxycortisol this procedure provides a more accurate estimation of *total* free 17,21-dihydroxy-20-ketosteroids in plasma. The quantitative recovery of 11-desoxycortisol is of considerable practical value, since it is a biosynthetic precursor of cortisol and its presence is being recognized with greater frequency in various spontaneous and induced abnormal adrenal states.

CURRENT methods for quantitating plasma levels of 17-hydroxycorticosteroids (17-OH-CS) in general utilize the color reaction first described by Porter and Silber (1). In this reaction, corticoids with 17, 21-dihydroxy-20-keto groups form phenylhydrazones which are yellow chromophores absorbing ultraviolet light maximally at 410 mμ (2). Although other nonsteroidal compounds may absorb similarly (3), the color reaction is presumed to measure principally cortisol,[1] the most abundant 17-OH-CS in the peripheral circulation of normal man (4). Recent interest in disorders of adrenocortical steroidogenesis, both congenital (5) and drug-induced (6–8), has directed attention toward the quantitative measurement of 11-desoxycortisol[2] as well. This compound, since it is structurally

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[1] Cortisol is 11β,17α,21-trihydroxy-Δ^4-pregnene-3,20-dione, also referred to as compound F (of Kendall) or F.

[2] 11-Desoxycortisol is 17α,21-dihydroxy-Δ^4-pregnene-3,20-dione, also referred to as compound S (of Reichstein) or S.

identical with cortisol except for the absence of an 11β-hydroxyl group, is also estimated by the Porter-Silber reaction (9). In initial experiments with the Peterson modification (10) of the method developed by Silber and Porter (11) it was found that excessive amounts of 11-desoxycortisol were lost. This observation prompted the present evaluation of the method and led to certain alterations in technique by which such losses may be avoided.

MATERIALS AND METHODS

1. The *reagents and equipment* used were essentially as specified by Peterson *et al.* (10).

2. *Cortisol and 11-desoxycortisol standards.*[3] Stock solutions of each in absolute ethanol contained 400 μg. per ml. and were stable for indefinite periods when stored at 4° C. From these, working solutions containing 20 μg. of steroid per ml. of absolute ethanol were prepared. Except when indicated, recovery studies were performed with 2.0 μg. of the standard evaporated to dryness from ethanol and dissolved in 10 ml. of distilled water. Water was used, rather than plasma, since fewer variables are involved. The recovery of cortisol from plasma is about 95 per cent of that from water (11).

3. Except for minor modifications, the procedure of Peterson *et al.* (10) was observed for the *extraction* of samples with dichloromethane as well as the subsequent *washes* of the solvent with water and 0.1 *N* NaOH.

4. Removal of dichloromethane:

(a) *By aspiration:* The procedure as adapted from Peterson *et al.* (10) is as follows:

1) Five-tenths ml. of color reagent (phenylhydrazine-sulfuric acid-ethanol) was added to each centrifuge tube (for the "sample blanks," 0.5 ml. of blank reagent (sulfuric acid-ethanol) was used).
2) The tubes were shaken horizontally on a Kahn shaker for five minutes and centrifuged at 2500 r.p.m. for ten minutes.
3) The dichloromethane layer was then discarded by aspiration into a vacuum trap.

(b) *By evaporation:* The present modification substituted for (a) is as follows:

1) Each centrifuge tube was placed in a water bath maintained at 37° to 40° C. and evaporated to dryness under a stream of room air filtered through glass wool.
2) The walls of each tube were then carefully rinsed with 2–3 ml. of dichloromethane and the extracts brought to dryness again.
3) The dry residue was dissolved in 0.5 ml. of dichloromethane. Five-tenths ml. of color reagent was added to one tube ("sample") of each pair and 0.5 ml. of blank reagent to the other ("sample blank").
4) Each tube was swirled by hand, placed in a semihorizontal position on the mechanical shaker and shaken for ten minutes.
5) After the tubes had been centrifuged for five minutes at 2500 r.p.m., the 0.5-ml. layer of dichloromethane was discarded by careful aspiration.

5. Quantitation.

(a) *Colorimetry:*

1) After removal of the solvent by either of the foregoing procedures, the tubes

[3] Supplied through the courtesy of Dr. Elmer Alpert, Merck Sharpe & Dohme, West Point, Pa.

were placed in a covered water bath at 60° C. for thirty minutes.

2) Upon removal, they were allowed to cool under running tap water for five minutes.

3) After transfer of the samples to silica cell microcuvettes, the absorbance of each was compared against that of distilled water at each of five wave lengths (370, 390, 410, 430 and 450 mμ).

(b) *Calculations:*

1) Estimation of absorbance due primarily to the phenylhydrazones of F and S.

a) Corrected absorbance of sample for extraneous chromogens which influence absorbance in both sample and sample blanks at *each wave length.*

Tubes treated with color reagent		*Tubes treated with blank reagent*	
Sample	(S)	Sample blank	(S_b)
Distilled water	(DW)	Distilled water blank	(DW_b)
Standard	(St)	Standard blank	(St_b)

1. Correction for absorbance due to sulfuric acid-ethanol (blank reagent) or to nonsteroidal chromogens in plasma which react with this reagent:

$$S - S_b = S_c$$
$$DW - DW_b = DW_c$$
$$St - St_b = St_c$$

2. Correction for absorbance due to other reagents (dichloromethane, phenylhydrazine, etc.):

$$S_c - DW_c = A_s$$
$$ST_c - DW_c = A_{st}.$$

b) Correction of absorption maximum (410 mμ) for extraneous chromogens which react with phenylhydrazine in sample tubes but have *linear* spectra from 370–450 mμ. The curves of the corrected absorbances for plasma samples containing little or no corticoids were nearly linear from 370–450 mμ, so that an adaptation of the Allen correction (12) was applied. Thus the corrected absorbance for the sample (S_{410}) at 410 mμ is given by:

$$S_{410} = A_s(410) \times 2 - [A_s(370) + A_s(450)].$$

A similar correction was applied for the standard (A_{st}) to derive St_{410}.

2) Calculation of concentration of phenylhydrazine-reacting corticoids:[4]

$$\frac{S_{410}}{St_{410}} \times 2 \times 1.0 \times \frac{100}{10} = \mu g. \text{ per } 100 \text{ ml. of plasma.}$$

RESULTS

When known quantities of compound S were added to water and proc-

[4] The data given for plasma samples (Tables 2, 3 and 4) are based upon 1.0 μg. of cortisol as a standard. This standard was used irrespective of whether the steroid measured was known to be cortisol or 11-desoxycortisol.

essed by Peterson's modification of the Silber-Porter method, only 24 per cent of the corticoid was recovered. The procedure was then systematically studied with reference to losses at various steps, as summarized in Table 1.

Color intensity due to cortisol and 11-desoxycortisol

The ultraviolet absorbance at 410 mμ was approximately the same for both F and S. This was determined by addition of phenylhydrazine-sulfuric acid-ethanol (color reagent) directly to measured amounts of each of the pure steroids which had been evaporated to dryness from the ethanol stock solutions. Both compounds conformed to Beer's law in an identical manner as shown by the linear relationship of absorbance to increases in molar concentration (Fig. 1).

The mean absorbances at 410 mμ found by direct colorimetry for 1.0-μg. quantities of cortisol and 11-desoxycortisol were 0.095 and 0.112, respectively. These values were taken to represent 100 per cent recovery in subsequent experiments concerned with other steps in the procedure.

Losses of steroid in dichloromethane: water partitions

Two steps in the method involve the partitioning of steroids between organic (solvent) and aqueous phases (water or plasma). The first of these, extraction of the free steroid from water with dichloromethane, resulted in small but significant losses of S and F (Table 1). The second partition, between dichloromethane and the alkaline and aqueous washes, yielded similar recovery values (Table 1). The combined steps involving solvent-water partitions resulted in loss of 11 to 23 per cent for each steroid, about 5 per cent of which could be accounted for by volume losses of dichloromethane throughout the procedure.

Losses of steroid in dichloromethane: color reagent partition

Evaluation of the partition of steroids between the color reagent and dichloromethane indicated a 46 per cent loss of S and virtually no loss of F (Table 1). When the volume of dichloromethane usually employed in this

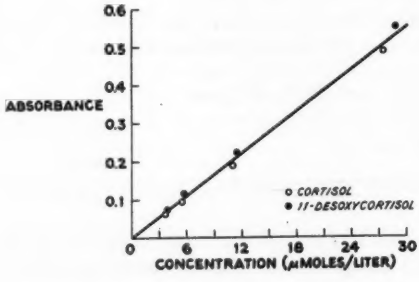

FIG. 1. Relationship of absorbance to concentration for 1.0-μg. quantities of cortisol and 11-desoxycortisol.

TABLE 1. LOSSES INCURRED AT VARIOUS STEPS IN THE DETERMINATION OF PLASMA FREE PHENYLHYDRAZINE-REACTING STEROIDS

Steps evaluated	11-Desoxycortisol		Cortisol		Reagents or procedures*						
	Loss (%)	No. of replicates	Loss (%)	No. of replicates	1	2	3	4	5 a	5 b	6
Colorimetry†	—	23	—	19	X						X
Water-dichloromethane extraction	11	3	4	4	X	X	X			X	X
Alkaline and aqueous washes	12	3	7	4	X		X	X		X	X
Dichloromethane-color reagent extraction	46	4	0	4	X		X		X		X
Dichloromethane evaporation	0	6	0	4	X		X			X	X
Total procedure											
Aspiration technique	76	11	24	17	X	X	X	X	X		X
Evaporation technique	11	8	16	8	X	X	X	X		X	X

* Key: 1. Dry steroid standard.
2. Distilled water.
3. Dichloromethane extraction.
4. Washes.
5. Removal of dichloromethane:
(a) By aspiration.
(b) By evaporation.
6. Color reagent.

† Mean absorbance of standards at 410 mμ (after Allen correction) taken as reference for 100%.

step (40 ml.) was reduced by evaporation, with no alteration in the volume of the color reagent (0.5 ml.), the recovery of S was progressively increased as the relative volumes of the two solvents were decreased from their initial ratio of 80:1. The complete evaporation of dichloromethane resulted in almost equal recoveries of both cortisol and 11-desoxycortisol (84 and 89 per cent, respectively) (Fig. 2).

Effect of evaporation of solvent upon absorption spectra of cortisol and 11-desoxycortisol

Comparison of absorbance at each of the five wave lengths routinely studied indicated that the absorption maximum for both steroids remained at 410 mμ and that no alteration or distortion of their spectra resulted from dichloromethane evaporation (Fig. 3).

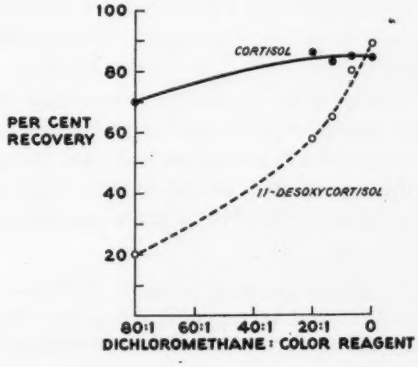

FIG. 2. Increases in recovery of standards obtained by decreasing the volume of dichloromethane extracted without altering the volume of color reagent.

Turbidity sometimes developed when the dry dichloromethane plasma residues were incubated directly with the color reagent. This could be avoided by returning the residue to solution with 0.5 ml. of dichloromethane before the phenylhydrazine reagent was added. The loss of F or S was not significantly increased at this ratio (1:1) of solvent to color reagent.

Effect of evaporation of solvent on estimation of cortisol and 11-desoxycortisol in plasma

The two procedures for removing the solvent were compared in the following clinical studies. A totally adrenalectomized patient was given infusions of cortisol and 11-desoxycortisol[5] on separate occasions. Blood samples taken at appropriate intervals were divided into two aliquots. One aliquot was analyzed by the modified method (evaporation of solvent), the

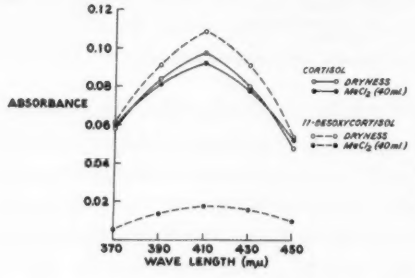

FIG. 3. Comparison of absorption spectra of standards after extraction from dichloromethane (40 ml.) with color reagent (0.5 ml.) and after evaporation to dryness from dichloromethane.

other by the original method (aspiration of solvent). During administration of *11-desoxycortisol*, the plasma 17-OH-CS levels in samples processed by the aspiration procedure ranged from 10 to 100 μg. per 100 ml. These values were more than doubled when evaporation was substituted for aspiration (Table 2). The levels found after infusion of *cortisol* were virtually the same with both techniques.

By use of dichloromethane evaporation, significantly higher concentrations of plasma Porter-Silber chromogens were regularly obtained when 11-desoxycortisol was present in the circulation, as in patients given SU-4885[6] (see Discussion), or 11-desoxycortisol (Table 3). Greater recoveries were also achieved in plasma taken from unselected patients with adrenocortical hyperplasia and carcinoma, conditions possibly associated with elevated levels of 11-desoxycortisol (see Discussion). Serial plasma samples drawn during infusion of SU-4885 in 2 subjects showed consistently higher levels of plasma 17-OH-CS when estimated with the modified technique (Table 4).

[5] Kindly provided by Dr. H. C. Peltier of The Upjohn Co., Kalamazoo, Michigan.

[6] SU-4885 is 1,2,-bis(3-pyridyl)-2-methylpropanone-1. It was generously supplied by Dr. C. H. Sullivan, CIBA Pharmaceutical Products Inc., Summit, New Jersey.

TABLE 2. DETERMINATION OF NONCONJUGATED PHENYLHYDRAZINE-REACTING STEROIDS IN PLASMA DURING INTRAVENOUS ADMINISTRATION OF COMPOUNDS S AND F TO A TOTALLY ADRENALECTOMIZED PATIENT

Plasma steroids during infusion of 11-desoxycortisol (S)			Plasma steroids during infusion of cortisol (F)		
Dichloromethane removed by		Loss* (%)	Dichloromethane removed by		Loss* (%)
aspiration	evaporation		aspiration	evaporation	
(μg./100 ml.)	(μg./100 ml.)		(μg./100 ml.)	(μg./100 ml.)	
91.9	211.0	57	172.0	170.2	None
91.2	192.3	53	196.5	—	—
20.9	62.3	66	96.4	98.6	2
28.7	58.0	51	35.6	40.0	11
8.4	23.8	63	21.8	25.1	13
12.4	36.7	66	47.9	48.2	1
Mean		59			5
Predicted†		65			8

* Percentage loss by aspiration technique based on results with evaporation technique as 100%.

† "Predicted" loss by aspiration: calculated by subtraction of percentage loss with evaporation from percentage loss with aspiration previously obtained with S and F standards carried through the *total procedure* (Table 1).

TABLE 3. DETERMINATION OF FREE PHENYLHYDRAZINE CHROMOGENS IN PLASMA IN VARIOUS CLINICAL CONDITIONS

Subject	Clinical condition	Plasma steroid values after dichloromethane removed by		Agent administered i.v.
		aspiration	evaporation	
		(μg./100 ml.)	(μg./100 ml.)	
S.T.	Hirsutism	14.1	24.1	SU-4885
B.S.	Normal	5.1	11.8	SU-4885
V.M.	Hirsutism	22.5	32.3	SU-4885
W.H.	Hypogonadism	10.3	22.2	SU-4885
A.N.	Adrenocortical carcinoma	33.3	50.1	None
B.P.	Adrenocortical hyperplasia	19.8	27.2	None
W.N.	Adrenocortical hyperplasia	104.0	132.0	ACTH
T.C.	Hypothyroidism	66.5	144.4	Compound S
C.D.	Portal cirrhosis	27.0	49.0	Compound S

DISCUSSION

A number of methods have been proposed in recent years for the estimation of nonconjugated plasma 17-hydroxycorticosteroids as Porter-Silber chromogens (10, 13–21). According to the results of the present study, not all of these would be applicable to the estimation of both 11-desoxycortisol and cortisol. One relatively simple and commonly used procedure, the Peterson modification (10) of the Silber-Porter method (11), was found to measure only one-fourth of the total quantity of 11-desoxycortisol present. However, the recovery of 11-desoxycortisol with this method could be increased threefold, approximating that of cortisol, by modifying the procedure so that color reagent was added directly to a dry residue of corticoids instead of using partition-extraction into the color reagent. Recoveries of cortisol, which remained essentially the same with this modified technique, were comparable to those reported by others (2). Fixed losses in the procedure, amounting to 10–20 per cent for both steroids, are commonly corrected for in the final calculations by values for the recovery of cortisol standards processed concurrently with plasma samples. By avoiding the *selective* loss of 11-desoxycortisol in the dichloromethane:color reagent partition, the modification presented allows the use of a single cortisol standard for this purpose, inasmuch as the absorbance of the phenylhydrazine derivatives of F and S were practically identical.

The practice of partitioning cortisol between dichloromethane and color reagent has been questioned previously because of the marked differences in volume of the two solvents (22). The present findings, in agreement with those of Peterson *et al.* (10), indicate that this factor is of little importance as far as the recovery of cortisol is concerned. Except for the present report, however, 11-desoxycortisol has not been similarly studied.

A review of the methods cited suggests that 11-desoxycortisol is not estimated uniformly by all of them. The method introduced by Nelson and Samuels in 1952 (13) and later modified (23) does measure this corticoid since, as reported by Migeon *et al.* (24), the recovery of 11-desoxycortisol was 80 per cent with this procedure. However, the disadvantages of expense and the time required with this technique have been pointed out by Eik-Nes (14). Nonetheless the problem emphasized by the present study was avoided, since the solvent phase (chloroform) was evaporated and the color reagent was then added directly to the dry residue.

No serious discrepancies between methods now in routine use have been noted previously in determinations on normal subjects, since cortisol accounts for at least 90 per cent of the nonconjugated Porter-Silber chromogens present in peripheral venous blood (25). The absence of detectable quantities of 11-desoxycortisol in *normal* subjects seems well established

(4). However, the appearance of this corticoid in an increasing number of *abnormal* states has been reported. Several cases of adrenocortical carcinoma have been described in which the urinary metabolites of 11-desoxycortisol exceeded those of cortisol (26, 27). Significant quantities of this corticoid have also been noted in Cushing's syndrome and in certain nonendocrine disorders, including hypertension, as well as in normal subjects given ACTH (27).

The study of enzymatic intra-adrenal defects in steroidogenesis has increased the interest in 11-desoxycortisol. The first description of the hypertensive form of congenital adrenal hyperplasia reported an elevated plasma level of Porter-Silber chromogens, none of which was found to be cortisol, but rather 11-desoxycortisol (28). It is of interest that the procedure employed by Eberlein and Bongiovanni in the study of this patient was that of Nelson and Samuels. Several nonhypertensive cases with similar findings, and presumably a lack of adrenocortical 11β-hydroxylase, have recently been reported (29). Considerable attention has lately been given to the chemical lesion produced by an 11β-hydroxylase inhibitor, SU-4885, which partially reproduces the congenital adrenal disorder described insofar as it causes the adrenal to secrete 11-desoxycortisol rather

TABLE 4. DETERMINATION OF NONCONJUGATED 17-HYDROXYCORTICOIDS (17-OH-CS) IN PERIPHERAL VENOUS PLASMA DURING INTRAVENOUS ADMINISTRATION OF SU-4885

Subject	Su-4885*	Hour	Plasma 17-OH-CS values after dichloromethane removed by	
			aspiration	evaporation
			(μg./100 ml.)	(μg./100 ml.)
W.H., male, aged 20	60 mg./Kg. over a 4-hour period	0	17.8	—
		2	5.4	18.0
		3	10.3	22.2
		4	6.5	13.6
B.S., female, aged 25	30 mg./Kg.† over an 8-hour period	0	20.2	17.9
		4	13.6	19.0
		8	15.7	23.6

* Doses are expressed in terms of SU-4885 ditartrate, of which 41.5% is the free base.

† A dose of 25 USP units of corticotropin was given in the infusion together with SU-4885. Corticotropin Injection (ACTH) was supplied through the courtesy of Dr. H. F. Hailman of The Upjohn Company, Kalamazoo, Michigan.

than cortisol (6). In our study, during the intravenous administration of SU-4885 the concentration of nonconjugated phenylhydrazine-reacting corticoids in peripheral venous plasma increased, but only when evaporation rather than aspiration was used for removal of dichloromethane (Table 4).

These selected examples illustrate that methods which measure 17,21-dihydroxy-20-ketosteroids as Porter-Silber chromogens require careful evaluation to assure that not only the absorbance but also the extraction of cortisol and 11-desoxycortisol are identical. The absence of uniformity between methods in this regard could give rise to serious discrepancies when the Porter-Silber chromogen being measured is not cortisol but 11-desoxycortisol. The modification presented allows the Silber-Porter method to be used equally well for the estimation of cortisol, 11-desoxycortisol, or a combination of both.

Acknowledgments

We are indebted to Satoshi Hane, B.S., for technical assistance and to Miss Mary Morrow for editorial advice. Stanley Kushinsky, Ph.D., University of Southern California School of Medicine, provided helpful suggestions with regard to certain of the theoretical considerations. The continued interest and critical evaluation of the material by Professor Peter H. Forsham, Director of the Metabolic Unit for Research in Arthritis and Allied Diseases, is gratefully acknowledged.

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Letter to the Editor

THE SYNDROME OF NONFUNCTIONAL OVARIES, GENITAL INFANTILISM AND NEGATIVE CHROMATIN PATTERN

To the Editor:

In the March 1959 issue of this Journal, Hutchings (1) reported a sexually immature 13-year-old girl with a male (negative) chromatin pattern who at laparotomy was found to have ovaries bilaterally. The author raised the question of the future sexual maturation of this patient. Our recent experience with 7 similar cases in girls beyond the normal age of puberty and in young women permits us to answer this question in part.

Our first 2 cases, reported one year ago in collaboration with H. Chimènes and Mme. J. Nathan-Kahn *et al.* (2), were not cited by Dr. Hutchings. A report on 5 additional cases has been published with P. Mauvais (3).

Analysis of the findings in our 7 cases indicated certain common characteristics which suggest a syndrome. All of these patients had a male chromatin pattern and bilateral ovaries normally situated. In addition, they exhibited infantile external genitalia, primary amenorrhea and practically no development of the breasts. The ovaries in each instance were conspicuously small, white and smooth and showed no evidence of ovulation. On histologic examination the appearance was similar to that described by Hutchings, namely, an ovary (Fig. 1) with a more or less sclerosed cortex, numerous primordial follicles, many atretic follicles, and no signs of ovulation. These ovaries were apparently nonfunctional, as indicated by a low level of excretion of folliculin, no detectable urinary pregnanediol, and lack of response to the administration of chorionic gonadotropin. The excretion of urinary gonadotropin was within the range of normal, which suggests that despite amenorrhea and a low excretion of folliculin, these ovaries have an inhibitory action on the hypophysis. In addition, in some instances, urinary 17-ketosteroid excretion was moderately high.

Important associated findings, especially anomalies, were also observed. Five of the 7 patients had aplasia of the labia minora and in 2 others these

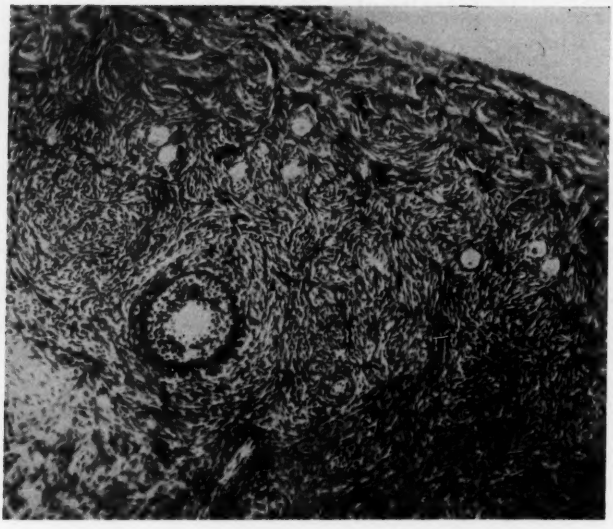

FIGURE 1

structures were hypoplastic. In 5 cases the patients were of short stature, and some were mentally defective. Among the other dystrophies noted were horseshoe kidney, harelip, mongoloid facies (2 cases), diencephalic obesity, pseudohypoparathyroidism, and epilepsy (2 cases).

Coelioscopy and determination of the excretion of urinary gonadotropin allows differentiation of this group of patients from those with typical Turner's syndrome (gonadal dysgenesis) and a male chromatin pattern.

Ford *et al.* (4) have recently described a sex chromosome anomaly in a case of gonadal dysgenesis with a male chromatin pattern. Their patient had 45 chromosomes and an XO sex chromosome constitution. It is possible that our cases were also XO and not XY. Whether or not the gonadal defect is related to an inversion of gonadal development (XY) or is a consequence of an abnormal genotype (XO), the disorder appears to be a chromosomal defect.

The ovaries of these "neutral" or "male" chromosomal patients are sterile and, like the testes in Klinefelter's syndrome, do not respond to chorionic gonadotropin and show progressive sclerosis.

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June 9, 1959

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The Endocrine Society

The 1960 Annual Meeting

The Forty-Second Annual Meeting of The Endocrine Society will be held in the Eden Roc Hotel, Miami Beach, Florida, Thursday, Friday and Saturday, *June 9, 10 and 11, 1960.*

The Chairman of Local Arrangements is Dr. Kenneth Savard.

Scientific Sessions will be held from 9:00 A.M. to 5:00 P.M. daily and in addition there will be simultaneous afternoon sessions. The annual dinner is scheduled for Friday, June 10, at 7:30 P.M. preceded by cocktails at 6:30 P.M.

All members are urged to make their hotel reservations immediately. The Eden Roc will hold 250 bedrooms and the adjoining hotels, the Montmartre Hotel and Lucerne Hotel, will hold 75 rooms each for members until May 1, 1960, after which time the hotel will not guarantee further reservations. Therefore it is imperative that you make your reservations early, directly with the hotel, advising them of time and date of arrival and departure. If you plan on remaining through June 12 for the American Medical Association and other meetings, it is imperative that your reservation be made from June 12 on, directly through the A.M.A. Housing Bureau, c/o Miami Beach Convention Bureau, 1700 Washington Avenue, Miami Beach, Florida, since no reservation beyond June 12 can be accepted directly by the hotels. Make your reservations early and avoid disappointment. Hotel reservation card will be mailed to members shortly after January 1, 1960.

Final program, membership card and advance registration forms will be sent on May 1, 1960 to members whose current dues have been paid.

Those wishing to present papers, which will be strictly limited to ten minutes, should send an original and 4 copies of the title and abstract to the Vice-President, Dr. Robert H. Williams, University of Washington School of Medicine, Seattle 5, Washington, *not later than February 15, 1960.* It is imperative that the abstracts be informative and complete with results and conclusions—not a statement that these will be presented at the meeting—in order that they may be of reference value and suitable for printing in the program. The reading and processing of approximately 200 abstracts submitted each year and compiling of the program from these

involves a tremendous amount of time and effort on the part of the Program Committee. THE COUNCIL REQUESTS THAT AUTHORS ADHERE STRICTLY TO THE FOLLOWING RULES WHEN SUBMITTING ABSTRACTS; OTHERWISE THEY CANNOT BE CONSIDERED:

1. IT IS ASSUMED THAT ABSTRACTS SUBMITTED FOR THIS PROGRAM HAVE NOT AND WILL NOT BE SUBMITTED ELSEWHERE.
2. Abstracts may not exceed 200 words, or equivalent space, exclusive of title. No footnotes or acknowledgments to sponsors can be published. References, if used, must be placed in the body of the text. The abstract should consist of a single paragraph, if possible. Structural chemical formula cannot be used.
3. The title heading must be arranged as follows:
   Line 1. Title, not to exceed 15 words;
   Line 2. Author/s. The name of each nonmember-author collaborating with member-authors is to be followed by the phrase "(by invitation)." Names of nonmembers who are introduced, *i.e.*, who are not collaborators with member-authors, are to be followed by the phrase "(introduced by . . .)." The principal degree, *e.g.*, M.D., of each author should be written after his name.
   Line 3. Institution of origin and city in which institution is located.
4. Authors who wish receipt of their abstracts acknowledged should enclose self-addressed post card with the title of the abstract noted thereon.
5. Authors are also to enclose 3×5 inch filing cards, one for each author, giving his name and initials in the upper right-hand corner, and the complete title of the abstract with all the authors listed in the center of the card. There should be a second card enclosed listing the title, all authors, and the mailing address of the senior author. This is necessary for indexing. Senior authors will be notified of the acceptance of their papers for the final program.
6. The body of the abstract, typed double space, should follow the heading. The original copy should be on bond paper. The original and 4 copies should be forwarded to Dr. Williams.
7. Abstracts should be letter perfect, since there will be no opportunity for proof reading by the authors.

The 1960 Awards

The selection of the recipients of the awards of The Endocrine Society is made by a Committee appointed by Council of the Society. These awards and fellowships carry no obligation by the recipient to the Society or to the donors.

Fred Conrad Koch Award

In 1957 a substantial legacy was bequeathed to the Society by the late Elizabeth Koch for the purpose of establishing the Fred Conrad Koch Memorial Fund in memory of her late husband, Distinguished Service Professor of Physiological Chemistry at the University of Chicago and pioneer in the isolation of the androgens. This is the highest honor of The Endocrine Society and is represented by a medal known as the Koch Medal of The Endocrine Society. The medal, as well as an honorarium of $3,500, is to be given annually to an individual for work of special distinction in endocrinology. The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada.

This Award was first presented in 1959, to Dr. W. W. Swingle. The Koch Medal has replaced the Medal of The Endocrine Society which was established in 1954 and presented to Dr. Carl R. Moore in 1955, Dr. Frederick L. Hisaw in 1956, Dr. Joseph C. Aub in 1957 and Dr. I. L. Chaikoff in 1958. The Medal of the Endocrine Society replaced the E. R. Squibb Award which was formerly the highest honor bestowed by the Society. Past recipients of the Squibb Award were Dr. George W. Corner in 1940, Dr. Philip E. Smith in 1941, Dr. Fred C. Koch in 1942, (no award in 1943), Dr. E. A. Doisy in 1944, Dr. E. C. Kendall in 1945, Dr. Carl G. Hartman in 1946, Drs. Carl F. and Gerty T. Cori in 1947, Dr. Fuller Albright in 1948, Dr. Herbert M. Evans in 1949, Dr. C. N. H. Long in 1950, Dr. J. B. Collip in 1951, Dr. James H. Means in 1952 and Dr. David Marine in 1953.

The Ciba Award

The Ciba Award, to recognize the meritorious accomplishments of an investigator not more than 35 years of age in the field of clinical or preclinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; 1945—Dr. Jane A. Russell; 1946—Dr. Martin M. Hoffman; 1947—Dr. Choh Hao Li; 1948—Dr. Carl Heller; 1949—Dr. George Sayers; 1950—Dr. Oscar M. Hechter; 1951—Dr. Albert Segaloff; 1952—Dr. Seymour Lieberman; 1953—Dr. Sidney Roberts and Dr. Clara Szego (Mrs. Roberts); 1954—Dr. Isadore M. Rosenberg; 1955—Dr. Jack Gross; 1956—

Dr. Alfred M. Bongiovanni; 1957—Dr. Nicholas S. Halmi; 1958—Dr. Monte A. Greer; 1959—Dr. Gordon L. Farrell. Prior to 1952 the Award was $1,200. It has now been increased to $2,500.

The Ayerst and the Squibb Fellowships

The Ayerst Fellowship was established in 1947 and the Squibb Fellowship in 1956. They are designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology. Each Fellowship is awarded on alternate years and the stipend, which will not exceed $5,000, may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree, or candidates for either of these degrees, are eligible for appointment.

Applicants must submit the following information:

1. Evidence of scientific ability as attested by studies completed or in progress.
2. Recommendations from individuals familiar with the candidate and his work.
3. A proposed program of study.
4. Acceptance of the individual by the head of the department in which the Fellowship will be held.
5. A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

The Upjohn Scholar of the Endocrine Society

The Council of The Endocrine Society has established a category of Scholars. A grant has been made available through the generosity of the Upjohn Company, and will be awarded to an established investigator or teacher in the field of endocrinology who wishes to extend the opportunities for work either in this country or abroad.

The award will not exceed $2,500 annually and will be granted on the basis of proposals submitted by the applicant. Such applications should include the estimated financial needs. The funds may be used for travel. maintenance and other expenses.

Nominations

Nominations for the Fred Conrad Koch Award, the Ciba Award, and the Ayerst and the Squibb Fellowships may be made by any member of The Endocrine Society. They should be submitted on forms which may be

obtained from the Office of the Secretary, 1200 North Walker Street, Oklahoma City 3, Oklahoma. Completed nominations should be returned to the Secretary not later than *October 15* each year.

Proposals for appointments as a Scholar of The Endocrine Society should be made in writing by the individual, and addressed to the Secretary of the Society. They should be submitted by *October 15* each year.

First International Congress of Endocrinology

The First International Congress of Endocrinology will be held in *Copenhagen, Denmark, July 18–23, 1960.*

United States members of the International Executive Committee are Dr. Warren O. Nelson and Dr. Leo T. Samuels, Dr. Gregory Pincus is Chairman of the Program Committee. The tentative program consists of ten symposia covering the general field of endocrinology plus round-table discussions and a number of submitted papers. Those desiring to submit papers should immediately contact Dr. Gregory Pincus, Worcester Foundation, Shrewsbury, Massachusetts. There will be a number of interesting scientific and technical exhibits.

In order to facilitate travel arrangements to the Copenhagen Convention, the Council of The Endocrine Society at its San Francisco meeting designated THOMAS COOK & SON as our authorized travel agents. They will in turn offer especially designed All Expense Paid Tours to Europe, including hotel reservations for the convention, as well as offering independent itineraries for any of our members who desire to travel separately by air or by steamship.

Registration is open to anyone interested in endocrinology at an approximate fee of $28 U.S. Members of the families of those attending may register as associates at an approximate fee of $14 U.S., fee to be paid at the time of registration or in advance to the American Express Company, which has been designated by the Executive Committee of the International Congress as official financial and travel agent. There will be a number of social functions and other entertainment provided for all registrants.

As you all know, European travel is unusually heavy during July and it is suggested that those contemplating attending the Congress should start making tentative reservations at the earliest possible moment by filling in and returning the card recently mailed to members, to THOMAS COOK & SON's special representative—Harry E. Kornbaum, Transportation Chairman, Rainbow Travel Service, 2817 Classen Boulevard, Oklahoma City 6, Oklahoma, who will correspond directly with each member about his individual travel arrangements to and from the Convention, at the same time keeping our National Officers fully informed of all travel arrangements

of its members. Completion of the card CARRIES NO OBLIGATION OR COMMITMENT ON YOUR PART. Send it in today for the complete travel arrangements for our Society.

The general expenses of a meeting of this magnitude are quite large and The Endocrine Society has made a substantial contribution from its treasury for organizational purposes. U.S. pharmaceutical firms have also contributed very generously. However, sufficient funds are not as yet available to assure the success of this meeting; therefore, *the committee suggests that each of our members make voluntary contributions to this fund.* Please make your checks payable to: The Endocrine Society—First International Congress of Endocrinology. Mark it *Contribution* and mail it to the office of the Secretary-Treasurer, 1200 North Walker Street, Oklahoma City 3, Oklahoma.

Program

The program of the First International Congress of Endocrinology which will be held in Copenhagen, Denmark, July 18–23, 1960, will consist of ten symposia and a round table discussion, along with sessions of communicated papers. The speakers at the symposia and the round table discussion have been invited by the Subcommittee on Program of the Congress. In Symposia #1–8 and in #10, the Subcommittee has also invited a discusser for each principal paper. Symposium #9 will consist of six principal papers and no discussers. The round table discussion will consist of eight sections, each with a principal speaker and three secondary speakers.

(For program see the December 1959 issue of the Journal.)

The American Goiter Association

1960 Annual Meeting and the 4th International Goiter Conference

The next Annual Meeting of the American Goiter Association will be held in *London, England, July 5–9, 1960,* at Church House, Dean's Yard, Westminster, London, S.W.1. This meeting, co-sponsored with the London Thyroid Club, will be the Fourth International Goiter Conference. For further information write Dr. Selwyn Taylor, Royal Society of Medicine, Dering Yard, 67c New Bond Street, London, W.1, England.

John C. McClintock, *Secretary,*
149½ Washington Avenue,
Albany 10, N.Y.

Training Program for Steroid Biochemistry

The Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts with the Department of Chemistry, Clark University, Worcester, Massachusetts and the Department of Biochemistry, College of Medicine, University of Utah, Salt Lake City, Utah announce the 1960–1961 Session of the Training Program for Steroid Biochemistry.

To alleviate the shortage of qualified trained personnel in steroid research, this Training Program will be sponsored by the United States Public Health Service through the National Cancer Institute of the National Institutes of Health. The courses will stress both theoretical and practical aspects as well as specialized laboratory techniques in steroid research.

Post-doctoral candidates (M.D. or Ph.D.) selected for admission both at Worcester and Utah will receive stipends of $5500 for a one-year period. Pre-doctoral candidates (B.S., M.S., or equivalent) selected for admission (only at Worcester) will receive stipends of $1800 for a six-month period.

Classes start *October 1, 1960.* Final date for completion of application: *June 1, 1960.*

Send inquiries and requests for applications to:

Dr. Kristen Eik-Nes,
Department of Biochemistry,
College of Medicine,
University of Utah,
Salt Lake City, Utah.

or

Dr. William R. Nes,
Department of Chemistry,
Clark University,
950 Main Street,
Worcester, Massachusetts.

Fourth National Cancer Conference

The Fourth National Cancer Conference will be held in *Minneapolis, Minnesota, September 13–15, 1960.* The theme will be "Changing Concepts Concerning Cancer." Correspondence should be addressed to the American Cancer Society, Inc., Medical Affairs Department, 520 West 57th Street, New York 19, N.Y.

Community Cancer Demonstration Project Grants

A sum of $1,500,000 has been appropriated by the Congress of the United States for fiscal year 1960 to further the widespread application of existing knowledge of preventing and controlling cancer. These funds will be administered by the Cancer Control Program, Public Health Service, under the technical guidance of the director of the National Cancer Institute.

Guidelines for the use of this money were developed by the Cancer Control Program's newly-formed Advisory Committee, which includes Doctors S. G. Taylor, III, (Chairman, Cancer Committee of the American

College of Physicians), Ulrich Bryner, Bernard Bucove, John W. Cline, Warren H. Cole, Joseph A. Cunningham, Harold S. Diehl, Lloyd M. Graves, John P. Lindsay, James J. Nickson, Mack I. Shanholtz, Charles E. Smith, John W. Spellman and David A. Wood.

Additional information may be obtained from the Cancer Control Program, Division of Special Health Services, Department of Health, Education, and Welfare, Washington 25, D. C., and from the eight regional offices of the U. S. Public Health Service.

International Society of Neurovegetative Research

The Annual Meeting of the International Society of Neurovegetative Research will be held in Freudenstadt/Schwarzwald, Germany, *July 29–31, 1960.* A Symposium will include the subjects of hypothalamic regulation of ACTH secretion and cerebral vegetative disorders. Communications regarding the meeting should be directed to Professor Dr. A. Sturm, Medizinische u. Nervenklinik der Stadtischen Kranken-Anstalten, Wuppertal-Barmen, Germany.

International Society of Cybernetic Medicine

This society, constituted in 1958 with the participation of 19 countries, held its first General Assembly in Naples in November 1959, and elected President: Prof. Aldo Masturzo; Vice-President: Prof. Paul Nayrac; Members of the Council: Profs. N. Wiener, G. Asboe-Hansen, F. Nember, A. Gata and C. Coruzzi. An International Symposium will be held in Naples in 1960, with the participation of Prof. Norbert Wiener. For information write to the Office of the Secretary-General, Via Roma 348, Naples, Italy.

Book Register

Ciba Foundation Study Group

No. 1. *Pain and Itch: Nervous Mechanisms.* In honor of PROF. MED. DR. Y. ZOTTERMAN, Department of Physiology, Kungl. Veterinärhögskolan, Stockholm, Sweden. Edited by G. E. W. WOLSTENHOLME and M. O'CONNOR for the Ciba Foundation. There were 20 participants in this Study Group and the subjects covered were: The significance of the peripheral anatomical arrangments of the nerves which serve pain and itch, the peripheral nervous mechanism of pain, the sensory functions of the non-myelinated afferent nerve fibers from the skin, a single unit analysis of cutaneous receptors with C afferent fibers, patterns of activity in the central sensory pathway, the thalamic and cortical reception of afferent impulses from the tongue, the peripheral mechanism of itch in man, and the mechanism of pain in trigeminal

neuralgia; 120 pages; 41 illustrations; 1959. Boston, Massachusetts; Little, Brown and Company. Price $2.50

No. 2. *Steric Course of Microbiological Reactions.* In honor of PROF. V. PRELOG, Laboratory of Organic Chemistry, Eidg. Technische Hochschule, Zürich, Switzerland. Edited by G. E. W. WOLSTENHOLME and C. M. O'CONNOR for the Ciba Foundation. Nineteen participants discussed: The steric course of microbiological reactions, the mechanism of hydrogen transfer with pyridine nucleotides, the steric mechanisms involved in the reactions of lactic acid, the steric and molecular specificity of steroid dehydrogenases, and the steric course of some microbiological and enzymic reductions of ketones; 116 pages; 37 illustrations; 1959. Boston, Massachusetts; Little, Brown and Company. Price $2.50.

L'Année Endocrinologique. Eleventh year. By M. ALBEAUX-FERNET, L. BELLOT, P. BRÉANT, P. BUGARD, J. CHABOT, J. DERIBREUX, M. GELINET and J.-D. ROMANI. A review of the most important recent advances in endocrinology, including studies on aldosterone, congenital adrenal hyperplasia, anomalies of thyroid hormone synthesis, pituitary coma, sexual abnormalities of the fetus due to administration of hormones during pregnancy, multiple endocrine syndromes, senescence, and steroid and thyroid therapy; 212 pages; 1959. Masson & Cie, Paris, France. Price 2,100 fr.

Report of the Medical Research Council for the Year 1957–1958. Great Britain. The report covers many items such as the development of research policy in laboratories in West Africa, various aspects of medical research (including mammalian fertilization), administrative matters, a summary of the work of the National Institute for Medical Research (including that of the Clinical Endocrinology Research Unit), a list of research fellowships, and a list of Council publications; 288 pages; 1959. London, Her Majesty's Stationery Office. Price 13s net.

Selected References 1952–58. Maternal Disorders Related to Fetal Stress, Perinatal Death and Congenital Defects. Public Health Bibliography Series No. 25. Contains 413 English language references listed under the headings, general, infectious diseases, metabolic disorders, blood disorders, neurologic disorders, collagen diseases, drug effects and statistical reports; 34 pages; 1959. A limited number of copies are available from the Library, National Institutes of Health, Bethesda 14, Maryland, Additional copies may be purchased from the Government Printing Office, Washington 25, D.C. for 15 cents per copy.